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(54) Title: METHODS OF REDUCING FACTOR VIII CLEARANCE AND COMPOSITIONS THEREFOR

(57) Abstract: The present invention provides methods of increasing the half-life of factor VIII. More specifically, the invention provides methods of increasing the half-life of factor VIII by substituting amino acids in the A2 domain or in the C2 domain of factor VIII or in both domains. It further provides factor VIII mutants produced by these methods. The invention also provides a method of using receptor-associated protein (RAP) to increase the half-life of factor VIII. The invention also provides polynucleotides encoding the mutant factor VIII, polynucleotides encoding RAP, and methods of treating hemophilia using the polypeptides and polynucleotides of the invention.

Methods of Reducing Factor VIII Clearance and Compositions Therefor

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Background of the Invention

Field of the Invention

This invention relates generally to a mutant factor VIII having increased half-life, methods of production, pharmaceutically acceptable compositions and uses thereof. This invention also relates to a method of using receptor associated protein to increase the half-life of factor VIII, methods of production, pharmaceutically acceptable compositions and uses thereof.

Related Art

Coagulation of blood occurs by either the "intrinsic pathway" or the "extrinsic pathway," whereby certain blood proteins interact in a cascade of proteolytic activations to ultimately convert soluble fibrinogen to insoluble fibrin. These threads of fibrin are cross-linked to form the scaffolding of a clot; without fibrin formation, coagulation cannot occur.

The intrinsic pathway consists of seven steps: (1) the proteolytic activation of factor XII; (2) activated factor XII cleaves factor XI to activate it; (3) activated factor XI cleaves factor IX, thereby activating it; (4) activated factor IX interacts with activated factor VIII to cleave and activate factor X; (5) activated factor X binds to activated factor V on a membrane surface, which complex proteolytically cleaves prothrombin to form thrombin; (6) thrombin proteolytically cleaves fibrinogen to form fibrin; (7) fibrin monomers assemble into fibrils, which are then cross-linked by factor XIII.

The extrinsic pathway consists of the following steps: (1) upon rupture of a blood vessel, factor VII binds to tissue factor, a lipoprotein present in tissues outside the vascular system; (2) factor VII is activated to factor VIIa by proteolytic cleavage; and (3) the factor VIIa-tissue factor complex cleaves and activates factor X. Thereafter, the extrinsic pathway is identical to the intrinsic pathway, *i.e.* the two pathways share the last three steps described above.

The plasma glycoprotein factor VIII circulates as an inactive precursor in blood, bound tightly and non-covalently to von Willebrand factor. Factor VIII (fVIII) is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor (vWf) and activates its procoagulant function in the cascade. In its active form, factor VIIIa (fVIIIa) functions as a cofactor for the factor X activation enzyme complex in the intrinsic pathway of blood coagulation, and it is decreased or nonfunctional in patients with hemophilia A.

In hemophilia, blood coagulation is impaired by a deficiency in certain plasma blood coagulation factors. People with deficiencies in factor VIII or with antibodies against factor VIII suffer uncontrolled internal bleeding that may cause a range of serious symptoms unless they are treated with factor VIII. Symptoms range from inflammatory reactions in joints to early death. The classic definition of factor VIII, in fact, is that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. A deficiency in vWf can also cause phenotypic hemophilia A because vWf is an essential component of functional factor VIII. In these cases, the half-life of factor VIII is decreased to such an extent that it can no longer perform its particular functions in blood-clotting.

The fVIII protein consists of a homologous A and C domains and a unique B domain which are arranged in the order A1-A2-B-A3-C1-C2 (Vehar, G.A., *et al.*, *Nature 312*:337-340 (1984)). It is processed to a series of Me^{2+} linked heterodimers produced by cleavage at the B-A3 junction (Fay, P.J., *et al.*, *Biochem. Biophys. Acta. 871*:268-278 (1986)), generating a light chain (LCh)

consisting of an acidic region (AR) and A3, C1, and C2 domains and a heavy chain (HCh) which consists of the Al, A2, and B domains (Fig. 1).

Activation of fVIII by thrombin leads to dissociation of activated fVIII (fVIIIa) from vWf and at least a 100-fold increase of the cofactor activity. The fVIIIa is a A1/A2/A3-C1-C2 heterotrimer (Fay, P.J., *et al., J. Biol. Chem* 266:8957-8962 (1991)) in which domains Al and A3 retain the metal ion linkage (Fig. 1) and the stable dimer A1/A3-C1-C2 is weakly associated with the A2 subunit through electrostatic forces (Fay, P.J., *et al., J. Biol. Chem* 266:8957-8962 (1991)). Spontaneous dissociation of the A2 subunit from the heterotrimer results in non-proteolytic inactivation of fVIIIa.

Infusion of fVIII/vWf complex or purified plasma or recombinant fVIII into patients with severe hemophilia A who do not have fVIII (Fijnvandraat, K., *et al., Thromb. Haemostas.* 77:298-302 (1997); Morfini, M., *et al., Thromb. Haemostas. 68*:433-435 (1992)) or in normal individuals (Over, J., *et al., J. Clin. Invest. 62*:223-234 (1978)) results in a similar fVIII disappearance with a half-life of 12-14 hours. Although the complex between fVIII and vWf is crucial for normal half-life and level of factor VIII in the circulation, the mechanisms associated with turnover of fVIII/vWf complex are not well defined.

The human factor VIII gene was isolated and expressed in mammalian cells (Toole, J. J., *et al., Nature 312*:342-347 (1984); Gitschier, J., *et al., Nature 312*:326-330 (1984); Wood, W. I., *et al., Nature 312*:330-337 (1984); Vehar, G. A., *et al., Nature 312*:337-342 (1984); WO 87/04187; WO 88/08035; WO 88/03558; U.S. Pat. No. 4,757,006), and the amino acid sequence was deduced from cDNA. Capon *et al.*, U.S. Pat. No. 4,965,199, disclose a recombinant DNA method for producing factor VIII in mammalian host cells and purification of human factor VIII. Human factor VIII expression in CHO (Chinese hamster ovary) cells and BHKC (baby hamster kidney cells) has been reported. Human factor VIII has been modified to delete part or all of the B domain (U.S. Pat. No. 4,868,112), and replacement of the human factor VIII B domain with the human factor V B domain has been attempted (U.S. Pat. No. 5,004,803). The cDNA

sequence encoding human factor VIII and predicted amino acid sequence are shown in SEQ ID NOs:1 and 2, respectively.

U.S. Patent No. 5,859,204, Lollar, J.S., describes mutants of human factor VIII having reduced antigenicity and reduced immunoreactivity.

Porcine factor VIII has been isolated and purified from plasma (Fass, D. N., *et al.*, *Blood 59*:594 (1982)). Partial amino acid sequence of porcine factor VIII corresponding to portions of the N-terminal light chain sequence having homology to ceruloplasmin and coagulation factor V and largely incorrectly located were described by Church, *et al.*, *Proc. Natl. Acad. Sci. USA 81*:6934 (1984). Toole, J. J., *et al.*, *Nature 312*:342-347 (1984) described the partial sequencing of the N-terminal end of four amino acid fragments of porcine factor VIII but did not characterize the fragments as to their positions in the factor VIII molecule. The amino acid sequence of the B and part of the A2 domains of porcine factor VIII were reported by Toole, J. J., *et al.*, *Proc. Natl. Acad. Sci. USA 83*:5939-5942 (1986). The cDNA sequence encoding the complete A2 domain of porcine factor VIII and predicted amino acid sequence and hybrid human/porcine factor VIII having substitutions of all domains, all subunits, and specific amino acid sequences were disclosed in U.S. Pat. No. 5,364,771 by Lollar and Runge, and in WO 93/20093. More recently, the nucleotide and corresponding amino acid sequences of the A1 and A2 domains of porcine factor VIII and a chimeric factor VIII with porcine A1 and/or A2 domains substituted for the corresponding human domains were reported in WO 94/11503. U.S. Patent No. 5,859,204, Lollar, J.S., discloses the porcine cDNA and deduced amino acid sequences.

Cellular endocytosis mediated by LRP was shown to be a mechanism of removal of a number of structurally unrelated ligands including several proteins related to coagulation or fibrilolysis. These ligands are: complexes of thrombin with antithrombin III (ATIII), heparin cofactor II (HC11) (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)), protease nexin I (Knauer, M.F., *et al.*, *J. Biol. Chem. 272*:12261-12264 (1997)), complexes of urokinase-type and tissue-

type plasminogen activators (u-PA and t-PA, respectively) with plasminogen activator inhibitor (PAI-1) (Nykjaer, A., *et al.*, *J. Biol. Chem. 267*:14543-14546 (1992); Orth, K., *et al.*, *Proc. Natl. Acad. Sci. 89*:7422-7426 (1992)), thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem. 272*:6784-6791 (1997)), tissue factor pathway inhibitor (TFPI) (Warshawsky, I., *et al.*, *Proc. Natl. Acad. Sci. 91*:6664-6668 (1994)), and factor Xa (Narita, M., *et al.*, *Blood 91*:555-560 (1998); Ho, G., *et al.*, *J. Biol. Chem 271*:9497-9502 (1996)).

LRP, a large cell-surface glycoprotein identical to α_2-macroglobulin receptor (Strickland, D.K., *et al.*, *J. Biol. Chem.* 265:17401-17404 (1990)), is a member of the low density lipoprotein (LDL) receptor family which also includes the LDL receptor, very low density lipoprotein (VLDL) receptor, vitellogenin receptor and glycoprotein 330 receptor. LRP receptor consists of the non-covalently linked 515 kDa α-chain (Herz, J., *et al.*, *EMBO J.* 7:4119-4127 (1988)) containing binding sites for LRP ligands, and the 85 kDa transmembrane β-chain. Within the α-chain, cluster of cysteine-rich class A repeats is responsible for ligand binding (Moestrup, S. K., *et al.*, *J. Biol. Chem 268*:13691-13696 (1993)). In contrast to the acidic ligand binding region in LRP, ligands of LRP expose regions rich in positively charged amino acid residues (Moestrup, S.K., *Biochim. Biophys. Acta 1197*:197-213 (1994)). This type of binding and 31 class A repeats present in LRP may be responsible for its wide ligand diversity and ability to serve as a multi-ligand clearance receptor. LRP is expressed in many cell types and tissues including placenta, lung and brain (Moestrup, S.K., *et al.*, *Cell Tissue Res. 269*:375-382 (1992)) and is a major endocytic receptor in the liver (Strickland, D.K., *et al.*, *FASEB J. 9*:890-898 (1995)).

A 39 kDa receptor-associated protein (RAP) binds to LRP with high affinity (K_d=4 nM (27)) and inhibits binding and LRP-mediated internalization and degradation of all ligands (Moestrup, S.K., *Biochim. Biophys. Acta* 1197:197-213 (1994); Williams, S.E., *et al.*, *J. Biol. Chem. 267*:9035-9040 (1992)), therefore serving as a useful tool for testing whether LRP is involved in endocytosis of a given ligand.

Severe hemophiliacs, who number about 10,000 in the United States, can be treated with infusion of human factor VIII, vWf/factor VIII complex or vWf which will restore the blood's normal clotting ability if administered with sufficient frequency and concentration. However, supplies have been inadequate and problems in therapeutic use occur due to difficulty in isolation and purification, immunogenicity, and the necessity of removing the AIDS and hepatitis infectivity risk.

Several preparations of human plasma-derived factor VIII of varying degrees of purity are available commercially for the treatment of hemophilia A. These include a partially-purified factor VIII derived from the pooled blood of many donors that is heat- and detergent-treated for viruses but contains a significant level of antigenic proteins; a monoclonal antibody-purified factor VIII that has lower levels of antigenic impurities and viral contamination; and recombinant human factor VIII, clinical trials for which are underway. Unfortunately, human factor VIII is unstable at physiologic concentrations and pH, is present in blood at an extremely low concentration (0.2 μg/ml plasma), and has low specific clotting activity.

The problems associated with the commonly used, commercially available, plasma-derived factor VIII have stimulated significant interest in the development of a better factor VIII product. There is a need for a more potent factor VIII; a factor VIII that is stable at a selected pH and physiologic concentration; a factor VIII that is has a longer half-life in circulating blood.

Summary of the Invention

The present invention relates to a method of increasing the half-life of factor VIII. More specifically, the present invention relates to a mutant of factor VIII having reduced clearance from plasma.

In one embodiment, the mutant factor VIII has one or more amino acid substitutions in the A2 domain.

In a preferred embodiment, the substituted amino acid(s) are important for receptor-dependent clearance of factor VIII, such that the resulting mutant factor VIII has a longer (increased) circulating half-life.

In another embodiment, the mutant factor VIII has one or more amino acid substitutions in the C2 domain.

In a preferred embodiment, the substituted amino acid(s) are important for receptor-independent clearance of factor VIII, such that the resulting mutant factor VIII has a longer (increased) circulating half-life.

In yet another preferred embodiment, amino acid(s) important for receptor-dependent clearance in the A2 domain and amino acid(s) important for receptor-independent clearance in the C2 domain are substituted, such that the resulting mutant factor VIII has an increased circulating half-life.

The invention also relates to a method of using receptor associated protein (RAP) to increase the half-life of factor VIII. Further aspects of the invention include a method of producing factor VIII mutants having an increased half-life, pharmaceutically acceptable compositions thereof, and a method of treating factor VIII deficiency using mutant factor VIII of the invention and/or RAP.

Brief Description of the Figures

FIG. 1. Domain structure of fVIII and its fragments. The domain structure of mature fVIII protein is shown in line 1. The LCh acidic region is labeled as AR. Thrombin-cleaved LCh (A3-Cl-C2), heterotrimeric fVIIIa (A1/A2/3-Cl-C2) and heterodimer A1/A3-Cl-C2 are shown in lines 2, 3 and 4.

FIGs. 2A and 2B. The amino acid sequence of mature, B-domainless fVIII (SEQ ID NO:5; composed from GenBank Accession No. X01179). The A2 sequence within fVIII is underlined and the sequence of the LRP binding site (residues 484-509) within A2 is indicated with asterisks. The amino acid residues shown as one-letter amino acid abbreviations.

FIGS. 3A and 3B. The deduced amino acid sequence of full-length factor VIII (SEQ ID NO:2; from GenPep Accession No. CAA25619.1 and GenBank Accession No. X01179).

FIG. 4. The deduced amino acid sequence of RAP (SEQ ID NO:4; GenBank Accession No. M63959). The signal sequence (amino acids 1-34) is underlined and the LDL receptor binding region (amino acids 237-353) is indicated with asterisks.

FIGS. 5A and 5B. Binding of ^{125}I-fVIII to purified LRP by ligand competition assay. ^{125}I-fVIII (1 nM) was incubated for 1 h at 37°C in wells coated with LRP (●) or BSA (○) in the presence of increasing concentrations of unlabeled competitors, fVIII (●, ○) or vWf (Δ), *panel A*, and RAP (●, ○), *panel B*. In the experiment (Δ), ^{125}I-fVIII was preincubated with vWf for 30 min at 37°C, prior to its addition to the wells. Following incubation, the wells were washed and ^{125}I-fVIII binding was determined. Binding of ^{125}I-fVIII in the presence of unlabeled fVIII, vWf, or RAP is expressed as the percentage of ^{125}fVIII binding, when no competitor was added. Each point represents the mean value of triplicates and the error bars display the standard deviation. The curves show a best fit of the data to a model describing heterologous ligand displacement from a single class of binding sites using the program LIGAND.

FIG. 6. Effect of fragments of fVIII on its binding to LRP. ^{125}fVIII (1 nM) and increasing concentrations of unlabeled HCh (●), A2 (▲), LCh (○) or A1/A3-C1-C2 (Δ) were incubated with LRP as described in Fig. 5. Each point represents the mean value and the standard deviation of the triplicates. The data were fitted as in Fig. 5 to a model describing heterologous ligand displacement from a single class of binding sites with K_i values of 120 and 132 nM for HCh and A2, respectively.

FIGS. 7A and 7B. Effect of monoclonal antibodies and synthetic peptides on ^{125}fVIII binding to purified LRP. *Panel A*, ^{125}fVIII (1 nM) and increasing concentrations of mAbs 413 (●) or T5 (○) were added to LRP coated wells as described in Fig. 5. In the control experiment (Δ), ^{125}If-VIII and increasing

concentrations of mAb 413 were added to BSA coated wells. *Panel B*, ^{125}I-fVIII and increasing concentrations of synthetic peptides consisting of the A2 domain residues 484-509 (●) or 432-456 (○) were added to LRP coated wells. In the control experiment (Δ), ^{125}I-fVIII and increasing concentrations of the peptide 484-509 were added to BSA coated wells. In the panels A and B, binding of ^{125}I-fVIII in the presence antibodies or peptides is expressed as the percentage of its binding, when no competitor was added. The mean and standard deviation of the triplicate measurements are presented.

FIGS. 8A and 8B. Internalization and degradation of ^{125}I-fVIII/vWf complex by LRP-expressing (MEF) and LRP-deficient (PEA 13) fibroblasts. Wells containing 2x10 of each MEF (○, ●) or PEA 13 cells (Δ, ▲) were incubated with 1 nM ^{125}I-fVIII/vWf in the absence (closed symbols) or presence (opened symbols) of RAP (1 μM). ^{125}I-fVIII/vWf complex was prepared by incubation of ^{125}I-fVIII with unlabeled vWf at a molar ratio 1:50 for 30 min at 37°C. At the indicated times, the amounts of internalized ^{125}I-fVIII (*panel A*) and degraded ^{125}I-fVIII (*panel B*) by the MEF and PEA 13 fibroblasts were determined as described under Experimental Procedures. In the experiment (▽), degradation of ^{125}I-fVIII (1 nM) by MEF cells in the presence of (0.1 mM) chloroquine is shown. Each data point represents the mean and standard deviation of duplicate determinations.

FIGS. 9A and 9B. Comparison of internalization of isolated ^{125}I-fVIII and components of fVIII/vWf complex. Wells containing 2x10^5 of each MEF and PEA 13 cells were incubated with 1 nM of isolated ^{125}I-fVIII or 1 nM of fVIII/vWf complex formed by mixing either ^{125}I-fVIII (1 nM) with unlabeled vWf (50 nM) or ^{125}I-vWf (50 nM) with unlabeled fVIII (1 nM). Following incubation for 6 hours with MEF cells in the absence of RAP (open bars) or in the presence of 1 μM RAP (solid bars) or after incubation with PEA 13 cells (hatched bars) the amounts of internalized (*panel A*) and degraded (*panel B*) isolated ^{125}I-fVIII, and ^{125}I-fVIII or ^{125}I-vWf from the fVIII/vWf complex were determined as described

in Fig. 8. The data shown are an average of duplicate determinations ± standard deviation.

FIGS. 10A and 10B. The A2 domain of fVIII inhibits the internalization and degradation of ^{125}I-fVIII/vWf complex by MEF fibroblasts. One nM of ^{125}I-fVIII/vWf complex was prepared as in Fig. 8 and incubated with $2x10^5$ of MEF cells in presence of 1 μM of A2 (○), 1 μM of A1/A3-C1-C2 (Δ), or in the absence of any competitor (●). At the indicated times, the amounts of internalized (*panel A*) and degraded ^{125}I-fVIII (*panel B*) were determined as in Fig. 8. Each data point represents the mean and standard deviation of duplicate determinations.

FIGS. 11A-D. Internalization and degradation of ^{125}I-A2 by MEF fibroblasts and by LRP-expressing smooth muscle cells (SMC) and alveolar epithelial cells (T2). In the *panels A and B*, $2x10^5$ of MEF (○, ●) or PEA 13 cells (Δ, ▲) were incubated with 10 nM ^{125}I-A2 in the absence (closed symbols) or presence (opened symbols) of RAP (1 μM). At the indicated times, the amounts of internalized ^{125}I-A2 (*panel A*) and degraded ^{125}I-A2 (*panel B*) by the MEF and PEA 13 fibroblasts were determined as described in Fig. 8. In the experiment (▽), degradation of ^{125}I-A2 by MEF cells in the presence (0.1 mM) chloroquine is shown. Each data point represents the mean and standard deviation of duplicate determinations. In the *panels C and D*, ^{125}I-A2 (10 nM) was incubated for 4 h at 37°C in the wells containing $3x10^5$ SMC (solid bars) or T2 (open bars) cells in the presence or absence of RAP (1 mM). The amount of ^{125}I-A2 internalized (*panel C*) and degraded (*panel D*) by the cells was determined as in Fig. 8. The data shown are an average of duplicate determinations ± standard deviation.

FIGS. 12A and 12B. The effect of RAP on clearance of ^{125}I-A2 (A) or ^{125}I-fVIII/vWf (B) from plasma of mice. BALB/c mice were injected into the tail vein by sample containing ^{125}I-A2 (36 nM), *panel A*, or ^{125}I-fVIII/vWf (20 nM), *panel B*, in the absence (●) or presence (○) of RAP (267 μM). At indicated time points, blood (50 μl) was collected into 10 μl of 100 mM EDTA and an aliquot (50 μl) was counted for radioactivity. The percentage of ligand remaining in circulation was calculated considering radioactivity of the aliquot taken at 1 min

after injection as 100%. The clearance of each preparation was examined in two mice, and the data plotted represent the average value ± standard deviation.

Detailed Description of the Preferred Embodiments

"Factor VIII" (or "coagulation factor VIII"), as used herein, refers to a plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. A congenital X-linked deficiency of biologically active factor VIII results in Hemophilia A, a potentially life-threatening disorder. Unless otherwise specified or indicated, as used herein, "factor VIII" denotes any functional human factor VIII protein molecule in its normal role in coagulation, including any fragment, analog derivative or modified factor VIII. The human factor VIII cDNA nucleotide and full-length predicted amino acid sequences are shown in SEQ ID NOs:1 and 2, respectively. Human factor VIII peptides of the invention include full-length factor VIII, full-length factor VIII minus Met at the N-terminus, mature factor VIII (minus the signal sequence), mature factor VIII with an additional Met at the N-terminus, and/or factor VIII with or without a B domain. Factor VIII of the invention may also include porcine factor VIII. The cDNA and predicted amino acid sequences of the porcine factor VIII are disclosed in U.S. Patent No..859,204.

"Subunits" of factor VIII, as used herein, are the heavy and light chains of the protein. The heavy chain of factor VIII contains three domains, A1, A2, and B. The light chain of factor VIII also contains three domains, A3, C1, and C2. Factor VIII is synthesized as an approximately 300 kDa single chain protein with internal sequence homology that defines the "domain" sequence NH_2 -A1-A2-B-A3-C1-C2-COOH.

In a factor VIII molecule, a "domain", as used herein, is a continuous sequence of amino acids that is defined by internal amino acid sequence identity and sites of proteolytic cleavage by thrombin. Unless otherwise specified, factor VIII domains include the following amino acid residues: A1, residues

Ala1-Arg372; A2, residues Ser373-Arg740; B, residues Ser741-Arg1648; A3, residues Ser1690-Ile2032; C1, residues Arg2033-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the factor VIII light chain activation peptide.

A "B-domainless" factor VIII or "B (-)" factor VIII, or fragment of thereof, as used herein, refers to any one of the factor VIII mutants described herein that lacks the B domain. The amino acid sequence of mature, B (-) factor VIII as constructed from GenBank Accession No. X01179 is shown in Figure 2 (SEQ ID NO:5). B (-) factor VIII of the invention includes B (-) factor VIII with or without a signal sequence and with or without a Met at the N-terminus.

As used herein, a "mutant factor VIII or fragment thereof" or "factor VIII mutant or fragment thereof" is an active factor VIII molecule or fragment thereof comprising at least one amino acid substitution.

"RAP," as used herein, refers to the receptor-associated protein, also called the α_2 macroglobulin receptor-associated protein. RAP reduces receptor-dependent clearance of factor VIII. The human RAP deduced amino acid sequence is shown in Figure 4 (SEQ ID NO:4; GenBank Accession No. P30533). The RAP cDNA sequence is shown in SEQ ID NO:3 and GenBank Accession No. M63959. Mutant RAP proteins of the invention may have an amino acid substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or more positions of RAP. An amino acid substitution at "position" 327, for example, of RAP, refers to an amino acid substitution at amino acid 327 of the RAP amino acid sequence in GenBank Accession No. P30533.

By "amino acid substitution" is meant a substitution of one amino acid for one of the remaining 19 naturally occurring amino acids. By an amino acid substitution at any one of positions "484 to 509," for example, is meant an amino acid substitution any position in the range, including at positions 484 and 509. The mutant factor VIII or RAP proteins of the invention may have an amino acid

substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or more positions.

An amino acid substitution at "position" 499, for example, of factor VIII, refers to an amino acid substitution at position 499 according to the numbering system of Wood *et al.*, *Nature 312*:330-337 (1984).

"Half-life," as used herein, refers to the half-life of factor VIII in circulation, as determined in animals such as mice, for example, using the method of Examples 1 and 2. Factor VIII has a half-life of 12-14 hours. As provided herein, methods to increase the half-life of factor VIII would lead to a factor VIII half-life of longer than 12-14 hours.

"Receptor-dependant clearance," as used herein, refers to the receptor-mediated removal of factor VIII from circulation. As described in the examples, receptor-dependant clearance is exhibited by MEF cells, and is inhibited by RAP. Receptor-dependent clearance includes, but is not limited, to LRP-mediated clearance of factor VIII clearance. Additional receptors may be involved in receptor-dependent clearance.

"Receptor-independent clearance," as used herein, refers to the removal of factor VIII from circulation by means different from receptor-dependant clearance. RAP does not inhibit receptor-independent clearance.

"Factor VIII deficiency," as used herein, includes deficiency in clotting activity caused by production of defective factor VIII, by inadequate or no production of factor VIII, or by partial or total inhibition of factor VIII by inhibitors. Hemophilia A is a type of factor VIII deficiency resulting from a defect in an X-linked gene and the absence or deficiency of the factor VIII protein it encodes. A deficiency in vWf can also cause phenotypic hemophilia A because vWf is an essential component of functional factor VIII. In these cases, the half-life of factor VIII is decreased to such an extent that it can no longer perform its particular functions in blood-clotting.

"Plasma," as used herein, refers to the fluid, non-cellular portion of the blood of humans or animals as found prior to coagulation. It is distinguished from serum, which is obtained after coagulation.

"Pharmaceutically acceptable carrier," as used herein, refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

"Patient," as used herein, refers to human or animal individuals receiving medical care and/or treatment.

"Congenital deficiency," as used herein, refers to the condition of an individual that lacks, as a result of heredity, a compound found in normal individuals. Congenital deficiencies are permanent absent transplantation or genetic intervention, which at this time are not guaranteed cures.

"Acquired deficiency," as used herein, refers to the condition of an individual that lacks, as a result of a non-congenital influence, a compound found in normal individuals. Acquired deficiencies are frequently the transient result of other conditions or their treatment, but are nonetheless debilitating and life threatening.

A "fusion protein," as used herein, is the product of a gene in which the coding sequence for one protein is extensively altered, for example, by fusing part of it to the coding sequence for a second protein from a different gene to produce a gene that encodes the fusion protein. As used herein, a fusion protein is a subset of the factor VIII protein or RAP protein described in this application.

A "corresponding" nucleic acid or amino acid or corresponding sequence of either, as used herein, is one present at a site in a factor VIII or mutant factor VIII molecule or fragment thereof that has the same structure and/or function as a site in the factor VIII molecule of another species, although the nucleic acid or amino acid number may not be identical.

"Procoagulant activity," as used herein, refers to factor VIII coagulation activity exhibited in a human factor VIII assay.

"Specific activity," as used herein, refers to the activity that will correct the coagulation defect of human factor VIII deficient plasma. Specific activity is measured in units of clotting activity per milligram total factor VIII protein in a standard assay in which the clotting time of human factor VIII deficient plasma is compared to that of normal human plasma. One unit of factor VIII activity is the activity present in one milliliter of normal human plasma. In the assay, the shorter the time for clot formation, the greater the activity of the factor VIII being assayed. Mutant factor VIII has coagulation activity in a human factor VIII assay. This activity may be less than, equal to, or greater than that of either plasma-derived or recombinant human factor VIII.

"Polypeptides," "molecules" and "proteins," as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types.

It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present invention are, to name an illustrative few, acetylation, acylation,

ADP-ribosylation, amidation, PEGylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formulation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *Proteins – Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W.H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pp. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, Analysis for protein modifications and nonprotein cofactors, *Meth. Enzymol. 182*: 626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Post translational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992).

In general, as used herein, the term polypeptide encompasses all such modifications, particularly those that are present in polypeptides synthesized by expressing a polynucleotide in a host cell.

The invention also relates to fragments, "derivatives" and analogs of these polypeptides. The terms "fragment," "derivative" and "analog" when referring to the polypeptides of FIGS. 2, 3 or 4, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. A mutant, fragment

derivative or analog of factor VIII refers to a polypeptide that retains factor VIII procoagulant activity. A mutant, fragment derivative or analog of RAP refers to a polypeptide that retains the ability to reduce receptor-dependent clearance of factor VIII. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide. Fragments, derivatives and analogs are described in detail herein.

A fragment, derivative or analog of the polypeptide of the invention may be (i) one in which one or more of the amino acid residues includes a substituent group, or (ii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iii) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide. In certain preferred embodiments it is a recombinant polypeptide.

Further particularly preferred in this regard are mutants, analogs and fragments; and mutants and analogs of the fragments, having the defined activity and/or having the amino acid sequence of the polypeptides of FIGS. 2, 3 or 4.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single-and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising

DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia.

Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

Polynucleotides of the present invention may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the

aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing--including splicing and polyadenylation signals, for example--ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexahistidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA 86*: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell 37*: 767 (1984), for instance.

An "effective amount" of an agent, as used herein, is an amount of such agent that is sufficient to bring about a desired result, especially upon administration of such agent to an animal or human.

The term "administration" is meant to include introduction of polypeptides or polynucleotides of the invention into an animal or human by any appropriate means known to the medical art, including, but not limited to, injection, oral, enteral, transdermal and parenteral (e.g., intravenous) administration.

The term "pharmaceutically acceptable salt" is intended to include salts of the mutant factor VIII or RAP of the invention. Such salts can be formed from pharmaceutically acceptable acids or bases, such as, for example, acids such as sulfuric, hydrochloric, nitric, phosphoric, etc., or bases such as alkali or alkaline earth metal hydroxides, ammonium hydroxides, alkyl ammonium hydroxides, etc.

The term "pharmaceutically acceptable composition" is intended to include solvents, carriers, diluents, and the like, which are utilized as additives or vehicles to preparations of the mutant factor VIII or RAP of the invention so as

to provide a carrier or adjuvant for the administration of such compounds to patients (human or animal) in need of the same. Such additives can perform certain functions, such as, for example, provide the proper ionic conditions for administration, stabilize the mutant factor VIII or RAP against inactivation or degradation, and/or increase the half-life of the mutant factor VIII or RAP. A pharmaceutically acceptable composition is medically compatible with the host to which it is being administered.

The term "treatment" or "treating" is intended to include the administration of the pharmaceutically acceptable compositions of the invention comprising effective amounts of mutant factor VIII or RAP (polypeptides or polynucleotides) of the invention to a patient for purposes which may include prophylaxis, amelioration, prevention or cure of a medical disorder.

A material is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is normally and naturally found before such purification and those contaminants normally and naturally found with the substance *in vivo* or *in vitro* are substantially absent from the final preparation of the material. When administered to a subject in need of treatment, the mutant factor VIII or RAP of the invention is substantially free of natural contaminants which associate with the mutant factor VIII or RAP either *in vivo* (in the host from which the mutant factor VIII or RAP was isolated), or *in vitro* (as a result of a chemical synthesis). By "substantially absent" is meant that such contaminants are either completely absent or are present at such low concentrations that their presence (1) does not interfere with the desired therapeutic effect of the active agent in the therapeutically acceptable composition when such composition is administered to a patient in need of same and (2) does not harm the patient as the result of the administration of such composition.

Since current information indicates that the B domain has no known effect on factor VIII function, in some embodiments the B domain is deleted ("B domain (-)" or "B domainless") in the mutant factor VIII molecule or fragments

thereof ("B(-) factor VIII" or "B domainless factor VIII") prepared by any of the methods described herein.

Generation of mutant(s) with a prolonged lifetime may be a promising approach to increase the efficacy and reduce the cost of fVIII infusion therapy. The invention provides methods of increasing the half-life of factor VIII by mutating factor VIII, and further provides methods of increasing the half-life of factor VIII using receptor-associated protein (RAP).

Factor VIII Mutants: A2 Domain

A recombinant mutant factor VIII having reduced receptor-dependent clearance and/or reduced receptor-independent clearance, and/or having superior coagulant activity, compared to human factor VIII, may be less expensive to make than plasma-derived factor VIII and may decrease the amount of factor VIII required for effective treatment of factor VIII deficiency.

The present invention provides active recombinant mutant factor VIII molecules or fragments thereof comprising at least one amino acid substitution in the A2 domain, polynucleotides encoding these, methods of producing and isolating them, and methods for characterizing their coagulant and plasma clearance properties.

The A2 domain is necessary for the procoagulant activity of the factor VIII molecule. Studies show that porcine factor VIII has six-fold greater procoagulant activity than human factor VIII (Lollar, P., and E. T. Parker 266 J. Biol. Chem. 12481-12486 (1991)), and that the difference in coagulant activity between human and porcine factor VIII appears to be based on a difference in amino acid sequence between one or more residues in the human and porcine A2 domains (Lollar, P., *et al.*, 267 J. Biol. Chem. 23652-23657 (1992)).

In one embodiment, the invention provides a method of increasing the half-life of factor VIII by substituting amino acids in the factor VIII A2 domain. In another embodiment, the invention provides mutant factor VIII and fragments thereof, and the polynucleotides encoding same, which have an increased

circulating half-life than human factor VIII. The increased circulating half-life is due to a reduction in receptor-dependent clearance of factor VIII. As shown in the examples, amino acids in the factor VIII A2 domain interact with at least one receptor that mediates A2 clearance and factor VIII clearance from plasma.

Thus, factor VIII mutants of the invention include mutants with one or more substitutions within the A2 domain. In a preferred embodiment, the factor VIII mutants have an amino acid substitution at one or more positions from 484 to 509. This region includes the following sequence: NH_2- Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe -COOH.

In another preferred embodiment, the factor VIII mutants have an amino acid substitution at one or more of positions 484, 489, 490, 493, 496 or 499.

The amino acid at a particular position is substituted with any of the 19 other naturally occurring amino acids. A2 amino acid substitutions of the invention are those that inhibit the interaction of factor VIII with its clearance receptor(s). Thus, nonconservative A2 amino acid substitutions are preferred over conservative substitutions. Conservative amino acid substitutions include, for example, the substitution of an acidic amino acid with another acidic amino acid, a basic amino acid with another basic amino acid, a hydrophobic amino acid with a another hydrophobic amino acid, a polar amino acid with another polar amino acid, or an aromatic amino acid with another aromatic amino acid. Conservative amino acid substitutions are well known in the art.

Thus, an example of a conservative substitution is the substitution of Lys with Arg, while an example of a preferred nonconservative substitution is the substitution of Lys with Asp, Glu, Tyr, Asn, Gln, Thr, Ser, Cys, Trp, Phe, Pro, Met, Val, Leu, Ile, Trp, Gly or Ala.

Preferred A2 amino acid substitutions of the invention are the substitution of Lys or Arg with Leu, Ile or Val. Additional preferred A2 amino acid substitutions of the invention are the substitutions of Lys or Arg with Asp or Glu.

Further preferred amino acid substitutions of the invention are the substitution of Lys or Arg with Ala, Ser, Thr, Met or Gly.

In another embodiment, amino acids at positions outside 484-509 are substituted, such as at positions 480, 481, 482, 483, 510, 511, 512 or 513. Preferred substitutions at these positions are those that reduce receptor-dependent clearance of factor VIII, such as introducing bulky or negatively charged amino acids.

Specifically provided as an exemplary and a preferred embodiment is active recombinant human factor VIII having substituted amino acids in the A2 domain, the polynucleotide encoding it, and the methods of producing, isolating, and characterizing its activity. The methods by which this mutant is prepared can also be used to prepare active recombinant factor VIII or fragments thereof having substituted amino acids in domains other than A2. One skilled in the art will recognize that these methods also demonstrate how other recombinant mutant factor VIII molecules or fragments thereof can be prepared in which amino acids are substituted. Additionally, recombinant methods are described in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds. (1991); and Sambrook, J., *et al.*, Molecular Cloning. A Laboratory Manual.

Mutant factor VIII is prepared starting with human cDNA (Biogen, Inc.) encoding the factor VIII sequence. In a preferred embodiment, the factor VIII encoded by this cDNA includes domains A1-A2-A3-C1-C2, lacking the entire B domain, and corresponds to amino acid residues 1-740 and 1649-2332 of single chain human factor VIII (see SEQ ID NO:2), according to the numbering system of Wood *et al.*, 312 Nature 330-337 (1984).

The mutant factor VIII cDNA are cloned into expression vectors for ultimate expression of active factor VIII protein molecules in cultured cells by established techniques, as described by Selden, R.F., "Introduction of DNA into mammalian cells," in *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds (1991).

In a preferred embodiment, a cDNA encoding mutant factor VIII is inserted in a mammalian expression vector, such as ReNeo, to form a mutant factor VIII construct. Preliminary characterization of the mutant factor VIII is accomplished by insertion of the mutant cDNA into the mammalian expression vector and transient expression of the mutant protein in COS-7 cells. A determination of whether active protein is expressed can then be made. The expression vector construct is used further to stably transfect cells in culture, such as baby hamster kidney cells, using methods that are routine in the art, such as liposome-mediated transfection (Lipofectin™, Life Technologies, Inc.). Expression of recombinant mutant factor VIII protein can be confirmed, for example, by sequencing, Northern and Western blotting, or polymerase chain reaction (PCR). Mutant factor VIII protein in the culture media in which the transfected cells stably expressing the protein are maintained can be precipitated, pelleted, washed, and resuspended in an appropriate buffer, and the recombinant mutant factor VIII protein purified by standard techniques, including immunaffinity chromatography using, for example, monoclonal anti-A2-Sepharose™.

In a further embodiment, the mutant factor VIII comprising amino acid substitutions is expressed as a fusion protein from a recombinant molecule in which sequence encoding a protein or peptide that enhances, for example, stability, secretion, detection, isolation, or the like is inserted in place adjacent to the factor VIII encoding sequence. Established protocols for use of homologous or heterologous species expression control sequences including, for example, promoters, operators, and regulators, in the preparation of fusion proteins are known and routinely used in the art. (See *Current Protocols in Molecular Biology,* Ausubel, F.M., *et al.*, eds, Wiley Interscience, N.Y.)

Other vectors, including both plasmid and eukaryotic viral vectors, may be used to express a recombinant gene construct in eukaryotic cells depending on the preference and judgment of the skilled practitioner (see, for example, Sambrook *et al.*, Chapter 16). Other vectors and expression systems, including

bacterial, yeast, and insect cell systems, can be used but are not preferred due to differences in, or lack of, glycosylation.

The purified mutant factor VIII or fragment thereof can be assayed for amount and for coagulation activity by standard assays including, for example, the plasma-free factor VIII assay, the one-stage clotting assay, and the enzyme-linked immunosorbent assay using purified recombinant human factor VIII as a standard.

Recombinant mutant factor VIII protein can be expressed in a variety of cells commonly used for culture and recombinant mammalian protein expression. A preferred cell line, available from the American Type Culture Collection, Rockville, Md., is baby hamster kidney cells, which are cultured using routine procedure and media.

Any mutant factor VIII construct having an amino acid substitution at one or more positions in the A 2 domain as described can be assayed by standard procedures for coagulant activity and may be assayed for receptor-dependent clearance as described herein to identify mutant factor VIII molecules with enhanced coagulant activity and/or reduced receptor-mediated clearance. Mutant molecules may also be identified that have reduced coagulant activity compared to human or porcine factor VIII but also have reduced receptor-mediated clearance. One skilled in the art will recognize that mutant factor VIII molecules or fragments thereof having less, equal, or greater coagulant activity, compared to human or porcine factor VIII, is useful for treating patients who have a factor VIII deficiency. The methods described herein to prepare active recombinant mutant factor VIII with amino acid substitution(s) in the A2 domain can be used to prepare active recombinant mutant factor VIII protein with amino acid substitution(s) in the C2 domain or fragments thereof.

These molecules can be expressed in COS-7 cells and baby hamster kidney cells as described above. They can be purified to homogeneity using methods known in the art, such as heparin-Sepharose™ and immunoaffinity chromatography. Protein concentration can be estimated by absorption of

ultraviolet light at A_{280}, and the specific activity of the constructs can be determined by dividing coagulant activity (measured in units per ml by single stage clotting assay) by A_{280}. Human factor VIII has a specific activity of approximately 3000-4000 U/A_{280}, whereas porcine factor VIII has a specific activity of approximately 20,000 U/A_{280}. In a preferred embodiment, the coagulant mutant factor VIII has a specific activity of 3000 U/A_{280}. In a preferred embodiment, the coagulant mutant factor VIII has a specific activity of 3000 U/A_{280}. The a specific activity of mutant factor VIII may be anywhere in the range of 1000-20,000 U/A_{280}.

As described herein, site-directed mutagenesis techniques are used to identify mutant protein with coagulant activity that can be enhanced, equal to, or reduced, compared to human factor VIII, but preferably is enhanced. Oligonucleotide-directed mutagenesis can be used as described in Kunkel, T.A., *et al.*, *Meth. Enzymol. 204*:125-139 (1991).

The mutant factor VIII proteins of the invention may have an amino acid substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, twenty or more positions of factor VIII. The mutant factor VIII molecules of the invention may have amino acid substitutions in more than one domain, such as having an amino acid substitution both in the A2 domain and in the C2 domain.

The present invention contemplates that mutant factor VIII cDNA and protein can be characterized by methods that are established and routine, such as DNA sequencing, coagulant activity assays, mass by ELISA and by UV absorbency at 280 nm of purified mutant factor VIII, specific coagulant activity (U/mg), SDS-PAGE of purified mutant factor VIII, and the like. Other known methods of testing for clinical effectiveness may be required, such as amino acid, carbohydrate, sulfate, or metal ion analysis.

Factor VIII Mutants: C2 Domain

The same methods employed for preparing mutant human factor VIII having A2 domain amino acid substitution(s) can be used to prepare other recombinant mutant factor VIII protein and fragments thereof and the polynucleotides encoding these, such as mutant factor VIII having amino acid substitutions in the C2 domain.

Mutant human factor VIII molecules with amino acid substitution(s) in the C2 domain, which have reduced or no receptor-independent clearance can be identified. More specifically, the procedures can be the same or similar to those described herein for amino acid substitution in the A2 domain (by alanine scanning mutagenesis, site-directed mutagenesis, etc.,) substituting amino acids in the C2 domain of B (-) factor VIII; insertion into an expression vector, such as pBluescript; expression in cultured cells; and routine assay for coagulant activity and receptor-independent clearance.

In one embodiment, the invention provides mutant factor VIII and fragments thereof, and the polynucleotides encoding same, which have an increased circulating half-life than human factor VIII. The increased circulating half-life of mutant factor VIII is due to a reduction in receptor-independent clearance of factor VIII.

The C2 domain consists of amino acid residues 2173-2332. Within this 154 amino acid region, positions 2303-2332 are involved in both phospholipid binding and vWf binding. A synthetic peptide of factor VIII amino acids 2310-2320 (in which residues 2310 and 2320 are covalently linked) competes with factor VIII for phospholipid binding. A comparison of factor V, which does not bind vWf, and factor VIII reveals 5 amino acids within positions 2311-2319 that are unique to factor VIII. Although not being bound by any theory, these unique positions (Gln2311, Ser2312, Val2314, His2315 and Gln2316) are important for receptor-independent clearance, but are not critical for vWf binding.

Thus, one embodiment of the present invention is a mutant factor VIII having an amino acid substitution at one or more of positions 2173-2332 in the

C2 domain. In another preferred embodiment, the mutant factor VIII has an amino acid substitution at one or more positions 2311-2319 in the C2 domain.

The amino acid at a particular position is substituted with any of the 19 other naturally occurring amino acids. C2 amino acid substitutions of the invention are those that inhibit the interaction of factor VIII with phospholipid. Thus, nonconservative C2 amino acid substitutions are preferred over conservative substitutions. Conservative amino acid substitutions include, for example, the substitution of an acidic amino acid with another acidic amino acid, a basic amino acid with another basic amino acid, a hydrophobic amino acid with a another hydrophobic amino acid, a polar amino acid with another polar amino acid, or an aromatic amino acid with another aromatic amino acid. Conservative amino acid substitutions are well known in the art.

Thus, an example of a conservative substitution is the substitution of Leu with Ile or Val, while an example of a preferred nonconservative substitution is the substitution of Leu with Asp, Glu, Arg, Lys, His, Tyr, Asn, Gln, Thr, Ser, Cys, Trp, Phe, Pro, Met, Trp, Gly or Ala. One preferred substitution is Ala.

Additional embodiments of the present invention include a method of treating hemophilia by administering a C2 domain mutant of factor VIII, pharmaceutically acceptable compositions comprising a C2 domain mutant of factor VIII either alone or in combination with RAP, and polynucleotides encoding a C2 domain mutant of factor VIII.

Furthermore, the amino acid substitution(s) in the C2 domain can be combined with amino acid substitution(s) in the A2 domain, to produce a mutant factor VIII with increased half-life.

Receptor Associated Protein

A preferred embodiment of the present invention is directed to a method of increasing the half-life of factor VIII by administering RAP. Preferably, the RAP binds LRP, more preferably, the RAP has an increased affinity for LRP as compared to the naturally occurring RAP.

In another preferred embodiment of the present invention, RAP is a fragment, mutant or analog. Preferably, the RAP fragment, mutant or analog retains LRP binding activity. More preferably, the RAP fragment, mutant or analog has increased affinity for LRP as compared to the naturally occurring RAP.

In one embodiment, the RAP is a fragment having LRP binding activity. Such RAP fragments may comprise 10, 20, 30, 40, 50, 60, 75, 100, 125, 150, 175, 200, 250, 300 or 350 or more contiguous amino acids.

In one embodiment, RAP comprises amino acids 1 to 357 of Figure 4 (full-length RAP; amino acids -19 to 323 of SEQ ID NO:4). RAP contains a signal sequence 34 amino acids in length. Thus, in another embodiment, RAP comprises amino acids 35 to 357 of Figure 4 (mature RAP; amino acids 1 to 323 of SEQ ID NO:4.).

In another embodiment of the present invention, RAP contains an N-terminal or a C-terminal deletion, or a combination of N- and C-terminal deletions. N-terminal deletions often result in a protein with increased stability. Thus, for example, deleting between 1 and 50 amino acids from the N-terminus of mature RAP is useful to produce a more stable RAP. Therefore, additional embodiments of the present invention include, for example, RAP comprising amino acids 36-357, 37-357, 38-357, 39-357, 40-357, 41-357, 42-357, 43-357, 44-357, 45-357, 46-357, 47-357, 48-357, 49-357, 50-357, 51-357, 52-357, 53-357, 54-357, 55-357, 56-357, 57-357, 58-357, 59-357, 60-357, 61-357, 62-357, 63-357, 64-357, 65-357, 66-357, 67-357, 68-357, 69-357, 70-357, 71-357, 72-357, 73-357, 74-357, 75-357, 76-357, 77-357, 78-357, 79-357, 80-357, 81-357, 82-357, 83-357, 84-357 and 85-357 of Figure 4 (positions 1-323, 2-323, 3-323, 4-323, 5-323, 6-323, 7-323, 8-323, 9-323, 10-323, 11-323, 12-323, 13-323, 14-323, 15-323, 16-323, 17-323, 18-323, 19-323, 20-323, 21-323, 22-323, 23-323, 24-323, 25-323, 26-323, 27-323, 28-323, 29-323, 30-323, 31-323, 32-323, 33-323, 34-323, 35-323, 36-323, 37-323, 38-323, 39-323, 40-323, 41-323, 42-323,

43-323, 44-323, 45-323, 46-323, 47-323, 48-323, 49-323 and 50-323 of SEQ ID NO:4).

The LDL receptor binding domain encompasses amino acids 237 to 353 of Figure 4 (amino acids 203 to 319 of SEQ ID NO:4). Thus, a preferred embodiment of the present invention is RAP comprising amino acids 237 to 353 (amino acids 203 to 319 of SEQ ID NO:4).

Another embodiment of the present invention is a polynucleotide encoding RAP.

In another embodiment of the present invention, RAP or a polynucleotide encoding RAP is used to treat hemophilia either alone or in combination with a factor VIII mutant.

Additional embodiments of the present invention include pharmaceutically acceptable compositions comprising RAP alone or in combination with one or more factor VIII mutants.

Pharmaceutically Acceptable Compositions

Pharmaceutically acceptable compositions comprising mutant factor VIII or RAP, alone or in combination with appropriate pharmaceutical stabilization compounds, delivery vehicles, and/or carrier vehicles, are prepared according to known methods, as described in *Remington's Pharmaceutical Sciences* by E.W. Martin.

In one preferred embodiment, the preferred carriers or delivery vehicles for intravenous infusion are physiological saline or phosphate buffered saline.

In another preferred embodiment, suitable stabilization compounds, delivery vehicles, and carrier vehicles include but are not limited to other human or animal proteins such as albumin.

Phospholipid vesicles or liposomal suspensions are also preferred as pharmaceutically acceptable carriers or delivery vehicles. These can be prepared according to methods known to those skilled in the art and can contain, for example, phosphatidylserine/phosphatidylcholine or other compositions of

phospholipids or detergents that together impart a negative charge to the surface, since factor VIII binds to negatively charged phospholipid membranes. Liposomes may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the mutant factor VIII or RAP is then introduced into the container. The container in then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

Mutant factor VIII or RAP can be combined with other suitable stabilization compounds, delivery vehicles, and/or carrier vehicles, including vitamin K dependent clotting factors, tissue factor, and von Willebrand factor (vWf) or a fragment of vWf that contains the factor VIII binding site, and polysaccharides such as sucrose.

Mutant factor VIII can be stored bound to vWf to increase the shelf-life of the mutant molecule. Additionally, lyophilization of factor VIII can improve the yield of active molecules in the presence of vWf. Lyophilization can also improve the yield of RAP. Current methods for storage of human and animal factor VIII used by commercial suppliers can be employed for storage of mutant factor VIII or RAP. These methods include: (1) lyophilization of factor VIII in a partially-purified state (as a factor VIII "concentrate" that is infused without further purification); (2) immunoaffinity-purification of factor VIII by the Zimmerman method and lyophilization in the presence of albumin, which stabilizes the factor VIII; (3) lyophilization of recombinant factor VIII in the presence of albumin.

Additionally, factor VIII has been indefinitely stable at 4°C in 0.6 M NaCl, 20 mM MES, and 5 mM $CaCl_2$ at pH 6.0 and also can be stored frozen in these buffers and thawed with minimal loss of activity.

Methods of Treatment

Mutant factor VIII or RAP is used to treat uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage) in hemophiliacs with and without inhibitory antibodies and in patients with acquired factor VIII deficiency due to the development of inhibitory antibodies. The active materials are preferably administered intravenously.

Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity *in vitro* of purified and partially-purified forms of factor VIII is used to calculate the dose of factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the *in vivo* bleeding defect. There are no reported discrepancies between standard assay of novel factor VIII molecules *in vitro* and their behavior in the dog infusion model or in human patients, according to Lusher, J. M., *et al.*, *New. Engl. J. Med. 328*:453-459 (1993); Pittman, D. D., *et al.*, *Blood 79*:389-397 (1992), and Brinkhous *et al.*, *Proc. Natl. Acad. Sci. 82*:8752-8755 (1985).

Usually, the desired plasma factor VIII level to be achieved in the patient through administration of the mutant factor VIII is in the range of 30-100% of normal. In a preferred mode of administration of the mutant factor VIII, the composition is given intravenously at a preferred dosage in the range from about 5 to 50 units/kg body weight, more preferably in a range of 10-50 units/kg body weight, and most preferably at a dosage of 20-40 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 to 10 days or until the bleeding episode is resolved. See, e.g., Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions – Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII)," Ch. 153, 1453-1474, 1460, in *Hematology*, Williams, W. J., *et al.*, ed. (1990).

Administration of an effective amount of RAP will result in similar levels of factor VIII in patient blood as indicated above. Patients with inhibitors may require more mutant factor VIII, or patients may require less mutant factor VIII because of its higher specific activity than human factor VIII or increased plasma half-life. Likewise, patients may require more or less RAP, depending on RAP's binding affinity to LRP or other factor VIII clearance receptor, or depending on its stability in circulating blood. As in treatment with human or porcine factor VIII, the amount of mutant factor VIII or RAP infused is defined by the one-stage factor VIII coagulation assay and, in selected instances, *in vivo* recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Administration

In a preferred embodiment, pharmaceutically acceptable compositions of mutant factor VIII or RAP alone or in combination with stabilizers, delivery vehicles, and/or carriers are infused into patients intravenously according to the same procedure that is used for infusion of human or animal factor VIII.

The treatment dosages of mutant factor VIII or RAP composition that must be administered to a patient in need of such treatment will vary depending on the severity of the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the mutant factor VIII or RAP is included in the pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the mutant protein to stop bleeding, as measured by standard clotting assays.

Treatment can take the form of a single intravenous administration of the composition or periodic or continuous administration over an extended period of time, as required. Alternatively, mutant factor VIII or RAP can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time. Mutant factor VIII or RAP can also be used to treat uncontrolled bleeding due to factor VIII deficiency in hemophiliacs who have developed antibodies to human factor VIII.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogens, e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res. 15*:167-277 (1981) and Langer, *Chem. Tech. 12*: 98-105 (1982) or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers 22*:547-556 (19831)), non-degradable ethylene-vinyl acetate (Langer *et al.*, supra), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988). While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization can be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release blood factor compositions also include liposomally entrapped blood factor or antibody. Liposomes containing the claimed blood factor or antibody are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Pat. No. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type, the selected proportion being adjusted for the optimal blood factor therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556. Additionally, Giles, A. R., *et al. Brit. J. Hematol. 69*:491-497 (1988) describe the formulation of factor Xa in phosphatidylcholine-phosphatidylserine vesicles.

Additionally, mutant factor VIII or RAP can be administered by transplant of cells genetically engineered to produce the protein or by implantation of a device containing such cells, as described below.

Gene Therapy

Polynucleotides encoding the mutant factor VIII or RAP may be employed in accordance with the present invention by expression of such mutant factor VIII or RAP *in vivo*, in treatment modalities often referred to as "gene therapy."

Mutant factor VIII or RAP can also be delivered by gene therapy in the same way that human factor VIII can be delivered, using delivery means such as retroviral vectors. This method consists of incorporation of factor VIII cDNA into human cells that are transplanted directly into a factor VIII deficient patient or that are placed in an implantable device, permeable to the factor VIII molecules but impermeable to cells, that is then transplanted. The preferred method will be retroviral-mediated gene transfer. In this method, an exogenous gene (e.g., a factor VIII cDNA) is cloned into the genome of a modified retrovirus. The gene/cDNA is inserted into the genome of the host cell by viral machinery where it will be expressed by the cell. The retroviral vector is modified so that it will

not produce virus, preventing viral infection of the host. The general principles for this type of therapy are known to those skilled in the art and have been reviewed in the literature (e.g., Kohn, D.B., and P.W. Kantoff, *Transfusion 29*:812-820 (1989)).

Thus, for example, cells from a patient may be engineered with a polynucleotide, such as a DNA or RNA, encoding a polypeptide ex vivo, and the engineered cells then can be provided to a patient to be treated with the polypeptide. For example, cells may be engineered ex vivo by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention. Such methods are well-known in the art and their use in the present invention will be apparent from the teachings herein.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by procedures known in the art. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct then may be isolated and introduced into a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors herein above mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Such vectors well include one or more promoters for expressing the polypeptide. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al.*, *Biotechniques 7*: 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, RNA polymerase III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, A., *Human Gene Therapy 1*:5-14 (1990). The vector may be transduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line will generate infectious retroviral vector particles, which include the polynucleotide(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the polynucleotide(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent

to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

All patents, publications and publicly available sequences referred to herein are expressly incorporated by reference.

Examples

Example 1

Activated factor VIII (fVIIIa) functions in the intrinsic pathway of blood coagulation as a cofactor for factor IXa in the conversion of factor X to activated factor X (Xa). When IXa is bound to membrane and fVIII the rate of factor X to IXa conversion increases 100,000-1,000,000 fold. The procoagulant activity of fVIIIa is regulated by rapid and potentially reversible dissociation of the A2 subunit from the A1/A3C1C2 dimer and by activated protein C (APC) proteolysis of the residual fVIIIa. Removal of the A2 and A1/A3C1C2 fragments is an additional *in vivo* mechanism to control factor VIIIa activity at the site of blood coagulation.

We tested this in a model system using mouse embryonic fibroblasts (MEF) that express low density lipoprotein receptor related protein (LRP) a multi ligand endocytic receptor and PEA 13 fibroblasts that are genetically deficient in LRP. Using the above model system we studied the mechanisms of cellular uptake and degradation of thrombin activated fVIII subunits to evaluate the role of these mechanisms in regulation of fVIIIa level.

Methods

Cell mediated ligand internalization and degradation assays. Cells were seeded into 24 well dishes and allowed to grow for 24 hours at 37°C. 5% CO_2 MEF and PEA 13 cells were incubated for selected time intervals at 37°C with ^{125}I-labeled fVIIIa fragments in the presence and absence of unlabeled competitors as described in the figure legends. Radioactivity appearing in the cell culture medium that was soluble after precipitation with 10% trichloroacetic acid (TCA) was taken to represent degraded ligand. Total ligand degradation was corrected by subtracting the amount of 10% TCA soluble radioactivity occurred in control wells lacking cells. The amount of labeled ligand bound to the cell surface or that was internalized by cells was determined as follows. Cells were washed with cold phosphate buffered saline and treated with a trypsin EDTA proteinase K solution. Surface bound material was defined as the amount of radioactive ligand released by this treatment and the amount of internalized ligand was defined as the amount of radioactivity which remained associated with the cell pellet following the treatment.

Determining of the A2 affinity for LRP. LRP (3.5 µg/ml) in 0.1 M $NaHCO_3$, pH 9.6 was incubated in Immulon I microtiter well strips for 16 hours at 4°C. After washing with TBS, 5 mM $CaCl_2$, 0.05% Tween 20 buffer (TBS-T) and blocking with 3% BSA, ^{125}I-A2 (5 nM) and increasing concentrations unlabeled A2 (0-1750 nM) were added. Following the incubation for 1 hour at 37°C and washing with TBS-T, the radioactivity bound to the wells was counted. ^{125}I-A2 binding in the presence of unlabeled A2 was plotted using the computer program "Ligand." The K_d value for A2/LRP binding was calculated from the displacement curve, showing a best fit of the data to a single class of sites.

Effect of RAP on the clearance of ^{125}I-A2 domain from the plasma of mice. To elucidate the role of LRP receptor in the clearance of the A2 domain from

plasma *in vivo* we tested the plasma level of ^{125}I-labeled A2 in the presence and absence of RAP after tail vein injection in mice. 250 µl samples of A2 (36 nM), in the presence and absence of RAP (267 µM) were injected into the tail vein of BALB/c mice. At the indicated times, blood (50 µl) was collected into 10 µl of 0.5 M EDTA and counted for its ^{125}I content. RAP significantly delays the plasma elimination of A2 domain. This experiment indicates that a RAP dependent hepatic receptor, LRP, plays a major role in the removal of A2 from circulation.

LRP receptor mediated internalization and degradation of the ^{125}I-A2 domain by fibroblast cells. The cellular uptake and degradation of activated factor VIII fragments was studied using mouse embryonic fibroblast (MEF) cells expressing low density lipoprotein receptor - related protein (LRP), a multi ligand endocytic receptor, and PEA 13 cells represents fibroblasts lacking LRP. The fVIIIa subunits interaction with MEF and PEA 13 cells represent an adequate model for *in vivo* processes because fibroblast cells became exposed to coagulation site upon vascular injury. LRP mediated internalization and degradation of some proteins (Thrombin:ATIII complex and other complexes of thrombin with inhibitors, tissue factor pathway inhibitor involved in coagulation cascade is known.

^{125}I-A2 (10 nM) was incubated with cells for several times and amount of surface bound, internalized and degraded ^{125}I-labeled protein were determined as described under "Methods." The A2 domain was internalized and degraded by MEF cells but not by PEA 13 cells suggesting that expression of LRP receptor is required for these processes. The internalization and degradation of A2 was blocked by RAP, an inhibitor of LRP binding to its ligands.

Internalization of the ^{125}I-A2 and APC cleaved A2 domain, by LRP presenting MEF cells and control PEA 13 cells, lacking LRP. Inactivation of fVIIIa by APC leads to a cleavage of the A2 at Arg^{562}. Since cofactor activity

cannot be reconstituted from $A2_N/A2_C$ and Al/A3C1C2 dimer, we proposed that $A2_N/A2_C$ removal from circulation may occur by a mechanism different than that for intact A2. To examine the effect of proteolysis by APC on cellular internalization of the A2 domain, we compared the ^{125}I-A2 and ^{125}I-$A2_N/A2_C$ uptake by MEF and PEA 13 cells. We found that in contrast to A2 domain, the internalization of ^{125}I-$A2_N/A2_C$ is not mediated by LRP receptor.

Binding the A2 domain to the immobilized LRP. To the microtiter wells with immobilized LRP ^{125}I-A2 (5 nM) and increasing concentrations of unlabeled A2 (0-1750 nM) were added. After incubation for 1 hour at 37°C the wells were washed with TBS-T and radioactivity bound to the wells was counted. ^{125}I-A2 binding in the presence of unlabeled A2 is expressed as the percentage of ^{125}I-A2 binding, when no competitor was added. The data was analyzed using the computer program "Ligand". The K_d value for A2/ LRP binding calculated from the displacement data was 130 nM.

Internalization of ^{125}I-labeled Al/A3ClC2 and Al^{336}/A3ClC2 by fibroblast cells. We proposed that phospholipid binding site previously localized to the C2 domain of fVIII light chain mediates the cellular surface binding and internalization of Al/A3ClC2 and Al^{336}/A3ClC2 dimers. To test this hypothesis we determined internalization ^{125}I-Al/A3ClC2 and ^{125}I-Al^{336}/A3ClC2 by MEF cells in the presence and absence of anti-C2 domain monoclonal antibody NMC-VIII/5, which blocks the membrane binding sites of the C2 domain.

Wells containing $2x10^5$ MEF cells were incubated with 3 nM of ^{125}I-Al/A3ClC2 or 3 nM of ^{125}I-Al^{336}/A3ClC2 at 37°C in the presence or absence of 30 nM monoclonal antibody NMC-VIII/5. In the control experiments, PEA 13 cells lacking LRP were incubated as above with ^{125}I-Al/A3ClC2 and ^{125}I-Al^{336}/A3ClC2. At several times internalization of the dimers was described under "Methods."

Since internalization of both ^{125}I-A1/A3C1C2 and ^{125}I-A1^{336}/A3ClC2 dimers was completely inhibited hy monoclonal antibody NMC-VIII/5, that recognizes the membrane binding site of fVIII C2 domain, we concluded that membrane binding of C2 is a critical step required for internalization of the above dimers. The rate of internalization was similar for MEF and PEA 13 cells, which indicates that LRP receptor is not involved in this process.

Degradation of ^{125}I-A1/A3C1C2 and ^{125}I-A1^{336}/A3ClC2 by MEF cells. MEF cells were incubated with ^{125}I-A1/A3ClC2 (3 nM) or ^{125}IA1^{336}/A3ClC2 (3 nM) for 22 hours at 37°C in the presence and absence PAP (1 μM). The degradation of dimers was measured as described under "Methods".

The degradation of A1/A3ClC2 dimer is RAP dependent. In contrast, degradation of APC cleaved A1^{336}/A3ClC2 dimer is RAP independent and does not correlate with LRP expression.

Conclusions

The A2 domain was internalized and degraded by mouse embryonic fibroblasts (MEF) which are expressing low density lipoprotein receptor - related protein (LRP), a multi ligand endocytic receptor. The internalization and degradation of A2 was blocked by RAP, an inhibitor of LRP binding to its ligands. *In vivo* clearance studies in mice demonstrated that RAP inhibited the clearance of ^{125}I-A2 from circulation. The radioactivity was preferentially accumulated in liver in the absence but not in the presence of RAP. This indicate that a RAP sensitive hepatic receptor most likely LRP, plays a major role in the removal of ^{125}I-A2 from the circulation.

The phospholipid binding site previously localized to the C2 domain of fVIII light chain mediates the cellular membrane binding and internalization of A1/A3ClC2 and A1^{336}/A3ClC2 dimers.

LRP receptor does not participate in cellular uptake and degradation of fragments $A2_N/A2_C$ and $A1^{336}$/A3C1C2, produced by irreversible inactivation of fVIIIa by APC. A2 and A1/A3C1C2 fragments produced by reversible inactivation of fVIIIa are removed by LRP-mediated and LRP-independent mechanisms, respectively. LRP is involved in the regulation of coagulation processes *in vivo*, by removal of A2 domain and A1/A3C1C2 dimer, the fragments from which active factor VIIIa can be reconstituted.

Example 2

The plasma glycoprotein factor VIII (fVIII) serves as a cofactor for the factor X activation complex in the intrinsic pathway of blood coagulation. FVIII circulates in plasma in a tight noncovalent complex with its carrier protein von Willebrand factor (vWf). Although the complex formation of fVIII with vWf is critical for maintenance of a normal half-life and level of fVIII in circulation, the mechanisms associated with fVIII turnover are not well defined. In the present study, we found that catabolism of fVIII is mediated by the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP), a liver endocytic, receptor responsible for *in vivo* clearance of a number of structurally unrelated ligands. A specific binding between fVIII and LRP was demonstrated by homologous ligand competition experiments, where a K_d of 116 nM was determined for fVIII binding to LRP. A 39 kDa receptor-associated protein (RAP), an antagonist of ligand binding by LRP, completely inhibited fVIII binding to purified LRP. The region of fVIII involved in its binding to LRP was localized to the A2 domain residues 484-509, based on the ability of the isolated A2 domain and the synthetic A2 domain peptide 484-509 to prevent fVIII interaction with LRP. Since vWf did not inhibit fVIII binding to LRP, we proposed that LRP receptor may internalize fVIII from its complex with vWf. In agreement with this, mouse embryonic fibroblasts (MEF) that express LRP, but not fibroblasts genetically deficient in LRP (PEA 13), were able to internalize and

degrade ^{125}I-fVIII/vWf complex. The latter processes were competed by RAP and A2 subunit of fVIII, indicating that cellular internalization and subsequent degradation were mediated by interaction of the A2 domain of fVIII with LRP. MEF cells were not able to internalize ^{125}I-vWf from ^{125}I-vWf /fVIII complex. This indicates that vWf does not follow fVIII in the LRP-mediated pathway and dissociates from fVIII at the early stage of endocytosis. *In vivo* clearance studies of ^{125}I-fVIII/vWf complex in mice demonstrated that RAP prolonged the half-life of ^{125}I-fVIII in circulation by 2.5-fold, indicating that RAP-sensitive receptor, most likely LRP, is responsible for the plasma clearance of fVIII.

Introduction

The plasma glycoprotein factor VIII (fVIII) functions as a cofactor for the factor X activation enzyme complex in the intrinsic pathway of blood coagulation, and it is decreased or nonfunctional in patients with hemophilia A. The fVIII protein consists of a homologous A and C domains and a unique B domain which are arranged in the order A1-A2-B-A3-C1-C2 (Vehar, G.A., *et al.*, *Nature 312*:337-340 (1984)). It is processed to a series of Me^{2+} linked heterodimers produced by cleavage at the B-A3 junction (Fay, P. J., *et al.*, *Biochem. Biophys. Acta. 871*:268-278 (1986)), generating a light chain (LCh) consisting of an acidic region (AR) and A3, C1, and C2 domains and a heavy chain (HCh) which consists of the A1, A2, and B domains (Fig. 1).

Transplantational studies both in animals and in humans demonstrated that the liver hepatocytes are the major fVIII-producing cells (Lewis, J. H., *et al.*, *N. Engl. J. Med 312*:1189-1191 (1985); Bontempo, F. A., *et al.*, *Blood 69*:1721-1724 (1987)). Immediately after release into circulation, fVIII binds with high affinity (K_d < 0.5 nM (MacGregor, I.R., *et al.*, *Vox. Sang. 69*:319-327 (1995); Saenko, E.L. and Scandella, D., *J. Biol Chem 272*:18007-18014 (1995)) to its carrier protein vWf to form a tight, noncovalent complex, which is required for maintenance of a normal fVIII level in the circulation. Complex formation with

vWf stabilizes association of the LCh and HCh within fVIII molecule (Wise, R.J., *et al., J. Biol. Chem. 266*:21948-21955 (1991)) and prevents fVIII from C2-domain mediated binding to phospholipid membranes (Gilbert, G.E., *et al., J. Biol. Chem.* 267:1586115868 (1992)), activation by activated factor X (Koppelman, S.J., *et al., J. Lab. Clin. Med. 123*:585-593 (1994)) and from protein C-catalyzed inactivation (Fay, P.J., *et al., J. Biol. Chem 266*:2172-2177 (1991)). vWf comprises a series of high molecular weight, disulfide-bonded multimers with molecular weight values as high as 2 x 10^7 Da (Hoyer, L.W. and Shainoff, J.R., *Blood 55*:1056-1059 (1980)) and circulates in plasma at 10 μg/ml or 50 nM, assuming a molecular mass of 270 kDa for vWf monomers (Girma, J.-P., *et al., Biochemistry 25*:3156-3163 (1986)). Since the concentration of fVIII in plasma is approximately 1 nM (Wion, K., *et al., Nature 317*:726-730 (1985)), one fVIII molecule is bound per 50 vWf monomers (Vlot, A.J., *et al., Blood 85*:3150-3157 (1995)).

Activation of fVIII by thrombin leads to dissociation of activated fVIII (fVIIIa) from vWf and to at least 100-fold increase of the cofactor activity. The fVIIIa is a A1/A2/A3-C1-C2 heterotrimer (Fay, P.J., *et al., J. Biol. Chem 266*:8957-8962 (1991)) in which domains A1 and A3 retain the metal ion linkage (Fig. 1) and the stable dimer A1/A3-C1-C2 is weakly associated with the A2 subunit through electrostatic forces (Fay, P.J., *et al., J. Biol. Chem 266*:8957-8962 (1991)). Spontaneous dissociation of the A2 subunit from the heterotrimer results in non-proteolytic inactivation of fVIIIa.

Infusion of fVIII/vWf complex or purified plasma or recombinant fVIII into patients with severe hemophilia A who do not have fVIII (Fijnvandraat, K., *et al., Thromb. Haemostas.* 77:298-302 (1997); Morfini, M., *et al., Thromb. Haemostas. 68*:433-435 (1992)) or in normal individuals (Over, J., *et al., J. Clin. Invest. 62*:223-234 (1978)) results in a similar fVIII disappearance with a half-life of 12-14 hours. Although the complex between fVIII and vWf is crucial for normal half-life and level of fVIII in the circulation, the mechanisms associated with turnover of fVIII/vWf complex are not well defined. We proposed that

fVIII/vWf complex is eliminated from plasma via clearance receptor and tested the possibility that this receptor is low density lipoprotein related protein receptor (LRP). Cellular endocytosis mediated by LRP was shown to be a mechanism of removal of a number of structurally unrelated ligands including several proteins related to coagulation or fibrilolysis. These ligands are: complexes of thrombin with antithrombin III (ATIII), heparin cofactor II (HC11) (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)), protease nexin I (Knauer, M.F., *et al.*, *J. Biol. Chem. 272*:12261-12264 (1997)), complexes of urokinase-type and tissue-type plasminogen activators (u-PA and t-PA, respectively) with plasminogen activator inhibitor (PAI-1) (Nykjaer, A., *et al.*, *J. Biol. Chem. 267*:14543-14546 (1992);Orth, K., *et al.*, *Proc. Natl. Acad. Sci. 89*:7422-7426 (1992)), thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem. 272*:6784-6791 (1997)), tissue factor pathway inhibitor (TFPI) (Warshawsky, I., *et al.*, *Proc. Natl. Acad. Sci. 91*:6664-6668 (1994)), and factor Xa (Narita, M., *et al.*, *Blood 91*:555-560 (1998); Ho, G., *et al.*, *J. Biol. Chem 271*:9497-9502 (1996)).

LRP, a large cell-surface glycoprotein identical to α_2-macroglobulin receptor (Strickland, D.K., *et al.*, *J. Biol. Chem.* 265:17401-17404 (1990)), is a member of the low density lipoprotein (LDL) receptor family which also includes the LDL receptor, very low density lipoprotein (VLDL) receptor, vitellogenin receptor and glycoprotein 330 receptor. LRP receptor consists of the non-covalently linked 515 kDa α-chain (Herz, J., *et al.*, *EMBO J.* 7:4119-4127 (1988)) containing binding sites for LRP ligands, and the 85 kDa transmembrane β-chain. Within the α-chain, cluster of cysteine-rich class A repeats is responsible for ligand binding (Moestrup, S. K., *et al.*, *J. Biol. Chem 268*:13691-13696 (1993)). In contrast to the acidic ligand binding region in LRP, its ligands expose regions rich in positively charged amino acid residues (Moestrup, S.K., *Biochim. Biophys. Acta 1197*:197-213 (1994)). This type of binding and 31 class A repeats present in LRP may be responsible for its wide ligand diversity and ability to serve as a multi-ligand clearance receptor. LRP is expressed in many cell types and tissues including placenta, lung and brain (Moestrup, S.K., *et al.*, *Cell Tissue Res.*

269:375-382 (1992)) and is a major endocytic receptor in the liver (Strickland, D.K., *et al.*, *FASEB J. 9*:890-898 (1995)). A 39 kDa receptor-associated protein (RAP) binds to LRP with high affinity (K_d=4 nM (27)) and inhibits binding and LRP-mediated internalization and degradation of all ligands (Moestrup, S.K. *Biochim. Biophys. Acta 1197*:197-213 (1994); Williams, S.E., *et al.*, *J. Biol. Chem. 267*:9035-9040 (1992)), therefore serving as a useful tool for testing whether LRP is involved in endocytosis of a given ligand.

In the present study we demonstrated that fVIII specifically binds to LRP, and that LRP mediates the internalization and subsequent degradation of fVIII in cultured fibroblasts and appears to be responsible for *in vivo* clearance of fVIII from circulation. We also demonstrated that interaction of the A2 domain of fVIII with LRP is responsible for mediating catabolism of fVIII.

Experimental Procedures

Monoclonal Antibodies. The monoclonal antibodies (mAbs) C4 (epitope within the fVIII light chain residues 1670-1684 (Foster, P.A., *et al.*, *J. Biol. Chem 263*:5230-5234 (1988))). C5 (epitope within A1 residues 351-361) and T5 (epitope within the residues 701-740 (Fulcher, C.A., *et al.*, *J. Clin. Invest. 76*:117-124 (1985))) were kindly provided by Dr. Carol Fulcher (Scripps Clinic and Research Foundation, La Jolla, CA). The anti-A2 mAb 8860 was generously provided by Baxter/Hyland. Mab 413 (epitope within A2 domain residues 484-509 (Healey, *et al.*, J. F., *J. Biol. Chem 270*:14505-14509 (1995))) was prepared as described previously (Saenko, E.L., *et al.*, *J. Biol. Chem 269*:11601-11605 (1994)).

Proteins. LRP was isolated from human placenta as described (Ashcom, J.D., *et al.*, *J. Cell Biol. 110*:1041-1048 (1990)). Human RAP was expressed in bacteria and purified as described (Williams, S.E., *et al.*, *J. Biol. Chem. 267*:9035-9040 (1992)). FVIII was purified from therapeutic concentrates of

Method M, American Red Cross (Saenko, E.L., *et al.*, *J. Biol. Chem* *271*:27424-27431 (1996)). HCh and LCh were prepared from fVIII as described previously (Saenko, E.L. and Scandella, D., *J. Biol Chem* 272, 18007-18014 (1995)). Purification of the Al/A3-C1-C2 dimer and A2 subunit was performed using ion exchange chromatography of thrombin activated fVIII on a Resource S column (Pharmacia) (Fay, P.T., *et al.*, *J. Biol. Chem* 268, 17861-17866 (1993)). Residual A2 present in the Al/A3-Cl-C2 preparation was removed by its passage over an immobilized mAb 8860 column equilibrated in 20 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$.

Radiolabeling of fVIII and synthetic peptides. Prior to iodination fVIII and A2 were dialyzed into 0.2 M sodium acetate, 5 mM calcium nitrate, pH 6.8 (iodination buffer). Five μg of fVIII in 30 μl of iodination buffer were added to lactoperoxidase beads (Worthington Biochemical Corp.), 5 μl of $Na^{125}I$ (100 mCi/ml, Amersham), and 5 μl of 0.03% H_2O_2 (Mallincrodt) and incubated for 4 min. Free $Na^{125}I$ was removed by chromatography on a PD10 column (Pharmacia). The specific radioactivity of fVIII and A2 was 3.5-5 μCi/μg of protein. The activity of ^{125}I-fVIII determined in the one-stage clotting assay (3740 units/μg) was similar to that of unlabeled fVIII.

Solid-phase binding assays. Homologous and heterologous ligand displacement assays were performed as previously described (Williams, S.E., *et al.*, *J. Biol. Chem. 267*:9035-9040 (1992)). Microtiter wells were coated with purified LRP or BSA (3 μg/ml) in 50 mM Tr-is, 0. 15 M NaCl, pH 8.0, for 16 h and then blocked with 3 % BSA in TBS. Coated wells were incubated with ^{125}I-A2 or ^{125}I-fVIII in 20 mM Tris-buffered saline pH 7.4, containing 5 mM $CaCl_2$, 0.05 % Tween-20 in the presence or absence of unlabeled competitors for 1 h at 37°C. The radioactivity bound to the wells was counted using a γ-counter (Pharmacia). Affinity constants were derived from homologous and heterologous displacement

data using the computer program LIGAND (Munson, PT and Rodbard, D. *Anal. Biochem. 107*:220-239 (1980)).

Cell-mediated ligand internalization and degradation assays. A normal mouse embryonic fibroblast line (MEF) and a mouse embryonic fibroblast cell line that is genetically deficient in LRP biosynthesis (PEA 13) were obtained from Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX) and maintained as described (Willnow, T.E. and Herz, J., *J. Cell Sci. 107*:719-726 (1994)). Cells were seeded at $1x10^5$ cells/well and allowed to grow for 24 h at 37°C, 5% CO_2. Cellular internalization and degradation assays were conducted as described previously (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)). Internalization and degradation of the ^{125}I-labeled fVIII and A2 was measured after incubation for indicated time intervals at 37°C in 0.5 ml of Dulbecco's modified medium (Gibco BRL) containing 2% BSA. Internalization was defined as radioactivity that is resistant to release from cells by trypsin (50 μg/ml) and proteinase K (50 μg/ml) (Sigma) in a buffer containing 5 mM EDTA. This treatment was previously shown to release radioligand bound to cell surface (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)) and therefore the ligand remained associated with cells after this treatment was considered as internalized. Degradation was defined as radioactivity in the medium that is soluble in 10% trichloroacetic acid. The value of degradation was corrected for non-cellular mediated degradation by subtracting the amount of degradation products generated in parallel wells lacking cells.

Clearance of ^{125}I-A2 domain and ^{125}I-fVIII/vWf complex from mouse plasma. The complex of ^{125}I-labeled fVIII with vWf in the presence or absence of RAP (in a total volume 250 μl) was injected in a tail vein of BALB/C mice over a period of approximately 20 seconds. At selected time intervals following injection (1, 3, 6, and 18 min), blood (50 μl) was withdrawn from the orbital plexus into 10 μl of 100 mM EDTA, and the radioactivity of the aliquot was determined. The

percentage of ligand remaining in circulation was calculated considering radioactivity of the aliquot taken at 1 min after injection as 100%. The clearance of each preparation was examined in two mice and the results were averaged. At the end of experiment, animals were sacrificed, liver lobules and kidneys were excised and weighed, followed by measuring the radioactivity in these tissues.

Results

Factor VIII binds to LRP and its binding is prevented by RAP. The ability of fVIII to bind to LRP *in vitro* was examined in homologous displacement binding assay. In the assay, binding of ^{125}I-fVIII (1 nM) to purified LRP, but not to BSA-coated wells, was competed (> 90%) by excess of unlabeled fVIII (Fig. 5A). The quantitative data regarding fVIII interaction with LRP were derived from the homologous displacement of ^{125}I-fVIII by unlabeled fVIII, which was adequately described by a model containing a single class of fVIII binding sites with K_d of 116 nM. To elucidate whether fVIII in a complex with vWf is also able to bind to LRP, we tested the effect of vWf on ^{125}I-fVIII binding to immobilized LRP. In this experiment, ^{125}I-fVIII was preincubated with vWf for 30 min at 37°C to allow complex formation prior to its addition to LRP coated wells. As shown in Fig. 5A, ^{125}I-fVIII binding to LRP was not inhibited by vWf up to the concentration of 1000 nM, which is 20-fold higher than its concentration in plasma (50 mM (Vlot, A.J., *et al.*, *Blood 85*:3150-3157 (1995))). This indicates that the complex formation with vWf does not affect fVIII ability to bind to LRP.

RAP, the antagonist of LRP-ligand binding, completely inhibited the binding of ^{125}I-fVIII to LRP-coated wells with K_i of 2.5 nM (Fig. 5B), a value similar to the previously determined affinity (4 nM) of RAP for LRP (Strickland, D.K., *et al.*, *J. Biol. Chem. 265*:17401-17404 (1990)). Together, these results demonstrate specific fVIII binding to LRP.

The amino acid residues 484-509 within the fVIII A2 domain are responsible for fVIII binding to purified LRP. In order to localize fVIII region(s) involved in interaction with LRP, binding between ^{125}I-fVIII and immobilized LRP was competed by unlabeled fVIII fragments. As shown in Fig. 6, HCh and A2 domain of fVIII, but not LCh (AR-A3-C1-C2) or A1/A3-C1-C2 dimer, displaced ^{125}I-fVIII from LRP in the heterologous ligand displacement assay. The K_i values determined for the HCh and A2 were similar, 120 nM and 132 nM, respectively. The similarity of the above K_d value for fVIII binding to LRP and the K_i value for inhibition of this binding by isolated A2 subunit indicates that A2 domain of HCh is responsible for fVIII binding to LRP.

To localize the region of the A2 domain responsible for the interaction with LRP, we tested the effect of anti-A2 monoclonal antibodies with known epitopes on fVIII/LRP binding. Fig. 7A shows that mAb 413 (epitope within the A2 domain residues 484-509 (Healey, J.F., *et al.*, *J. Biol. Chem 270*:14505-14509 (1995))) but not mAb T5 (epitope within the A2 domain residues 701-740 (35)) is able to block fVIII/LRP interaction. The concentration of mAb 413 required for 50% inhibition of ^{125}I-fVIII/LRP binding was 2.5 nM. The low molar excess (2.5-fold) of mAb 413 over fVIII required for 50% inhibition of fVIII/LRP binding is consistent with a previously reported high affinity of mAb 413 for fVIII (Lollar, P., *et al.*, *J. Clin. Invest. 93*:2497-2504 (1994)). In a control experiment, mAbs C5 (epitope within A1 residues 351-361) and C4 (epitope within LCh residues 1670-1684 (Foster, P.A., *et al.*, *J. Biol. Chem 263*:5230-5234 (1988))) did not have any effect on fVIII binding to LRP (data not shown), which is consistent with the lack of participation of A1 and LCh in fVIII binding to LRP.

Since it was previously demonstrated that mAb 413 recognizes synthetic peptide with a human fVIII sequence 484-509 (Healey, J.F., *et al.*, *J. Biol. Chem 270*:14505-14509 (1995)), we tested if the region of the A2 domain encompassed by peptide 484-509 is involved in binding to LRP. As seen from Fig. 7B, the synthetic peptide 484-509, but not the control A2 peptide 432-456, inhibited fVIII binding to LRP in a dose-dependent fashion, indicating that the region 484-509

of the A2 domain contains critical residues for fVIII binding to LRP. In a control experiment, no binding of ^{125}I-fVIII to BSA-coated wells was observed in the presence of peptide 484-509 (Fig. 7B).

Internalization and degradation of ^{125}I-fVIII complex with vWf by cultured fibroblasts is mediated by LRP. Since the data presented above demonstrated specific interaction between fVIII and LRP, and vWf does not interfere with this interaction, we hypothesized that LRP may be also capable of mediating the cellular internalization of ^{125}I-fVIII from its complex with vWf. To examine this hypothesis, cellular uptake and degradation experiments were conducted in mouse embryonal fibroblasts (MEF) which express LRP and in PEA 13 fibroblasts that are genetically deficient in LRP (Willnow, T.E. and Herz, J. *J. Cell Sci. 107*:719-726 (1994)). The ^{125}I-fVIII/vWf complex was prepared by 30 min (37°C) incubation of ^{125}I-fVIII with vWf at their plasma concentrations of 1 nM and 50 nM, respectively. As shown in Figs. 8A and B, MEF cells, but not PEA 13 cells lacking LRP, were capable of internalizing and degrading of ^{125}I-fVIII in the presence of vWf. Further, internalization and degradation of ^{125}I-fVIII by MEF but not by PEA 13 fibroblasts was inhibited by RAP, an antagonist of ligand binding to LRP. The ability of RAP to block the uptake and degradation of ^{125}I-fVIII/vWf in MEF cells and inability of PEA 13 cells to efficiently mediate uptake and degradation indicates that LRP is the mediator of ^{125}I-fVIII/vWf catabolism. To further characterize the degradation pathway of fVIII in the MEF cells, we tested the effect of chloroquine (an agent that blocks lysosomal degradation) on ^{125}I-fVIII degradation. As seen from Fig. 8B, the degradation of ^{125}I-fVIII is completely inhibited by chloroquine.

To elucidate if fVIII internalization in the absence of vWf is also mediated by LRP, we measured the internalization and degradation of isolated ^{125}I-fVIII (Fig. 9). As seen from Figs. 9A and B, both internalization and degradation of isolated ^{125}I-fVIII by MEF fibroblasts is approximately 2-fold higher than that in the presence of vWf. RAP inhibited internalization and degradation of ^{125}I-fVIII

to a lesser degree than those of ^{125}I-fVIII/vWf complex and, in addition, LRP-deficient PEA 13 fibroblasts were able to internalize and degrade isolated ^{125}I-fVIII. This indicates that LRP-mediated pathway is not the sole mechanism of fVIII internalization and degradation in the absence of vWf.

To determine whether vWf bound to fVIII is also internalized and degraded by MEF cells, internalization and degradation of ^{125}I-labeled vWf complexed with fVIII was measured. As shown in Figs. 9A and B, the amounts of internalized and degraded ^{125}I-vWf by both MEF and PEA 13 cells were less than 5 % of the corresponding amounts of ^{125}I-fVIII catabolized from its complex with vWf under the same experimental conditions. This indicates that vWf does not follow fVIII in the LRP-mediated pathway and possibly dissociates from fVIII at early stage of endocytosis, prior to entry of the complex into endosomal compartments.

The A2 subunit of fVIII inhibits endocytosis and degradation of ^{125}I-fVIII/vWf by MEF cells. Since we have demonstrated above that the A2 subunit of fVIII prevents an *in vitro* interaction between LRP and fVIII, we examined if A2 can also inhibit LRP-mediated internalization and degradation of fVIII/vWf complex by MEF cells. Figs. 10A and B demonstrate that 1000-fold excess of the A2 subunit over ^{125}I-fVIII/vWf complex effectively inhibit internalization (by >70% after 4 hours) and degradation (by >60% after 4 hours) of this complex. In contrast, A1/A3-C1-C2 heterodimer, which did not inhibit fVIII interaction with purified LRP in the above experiments, did not have any effect on ^{125}I-fVIII endocytosis and degradation by MEF cells (Fig. 10).

To confirm that the inhibitory effect of the A2 subunit results from its direct competition with ^{125}I-fVIII/vWf complex for LRP-mediated internalization and degradation, we tested whether MEF cells are able to internalize and degrade isolated A2 subunit. As shown in Figs. 11A and B, ^{125}I-A2 is readily internalized and degraded by LRP-expressing MEF cells. Both the internalization and degradation of the ^{125}I-labeled A2 were blocked in the presence of RAP. In

contrast, LRP-deficient PEA 13 cells were unable to internalize or degrade ^{125}I-A2 (Fig. 11), confirming that catabolism of the A2 subunit is LRP-mediated.

To verify that LRP-mediated internalization and degradation of the A2 domain was not the unique feature of the MEF cells, we tested ^{125}I-labeled A2 internalization and degradation by smooth muscle cells (SMC) and alveolar epithelial cells (T2), which also express LRP on their surfaces (Moestrup, S.K., *Cell Tissue Res. 269*:375-382 (1992)). As shown in Figs: 11C and D, RAP effectively inhibited both internalization of ^{125}I-A2 by SMC and T2 (by 81 % and 64 %, respectively), and its degradation (by 78 % and 68 %), indicating that these processes were mediated by LRP.

Thus, the data shown in Figs. 10 and 11 demonstrate that LRP is capable of binding fVIII via its A2 domain and of mediating fVIII endocytosis leading to lysosomal degradation.

Effect of RAP on the plasma clearance of ^{125}I-fVIII and ^{125}I-A2. To determine whether LRP is capable of catabolizing the isolated fVIII A2 subunit and whole fVIII from its complex with vWf *in vivo*, the effect of RAP on the clearance rates of ^{125}I-fVIII/vWf complex and ^{125}I-A2 in mice was tested. As shown in Fig. 12A, RAP increased the half-life of both ^{125}I-A2 and ^{125}I-fVIII in mouse plasma by approximately 4 and 2.5-fold, respectively. In addition, in the absence of RAP, most of radioactivity was found in the liver but not in kidney, consistent with LRP

presence in high abundance in hepatic tissues (Strickland, D.K., *et al.*, *FASEB J. 9*:890-898 (1995)). Thus, our data indicate that a RAP-sensitive hepatic receptor, LRP, plays a major role in the removal of fVIII and its A2 subunit from circulation.

Discussion

In the present study we demonstrated that LRP mediates the internalization and degradation of human fVIII in a model system using LRP-expressing cells and is responsible for fVIII clearance *in vivo*. This conclusion is based on several independent observations. First of all, we found that fVIII directly binds to purified LRP immobilized on microtiter wells, and that this binding is competed by RAP, an antagonist of ligands binding to LRP. Second, ^{125}I-fVIII is internalized from its complex with vWf by mouse fibroblasts expressing LRP (MEF cells) , but not by mouse fibroblasts genetically deficient in LRP (PEA 13 cells). Third, we demonstrated that RAP effectively inhibited the cellular uptake and degradation of ^{125}I-fVIII from its complex with vWf by MEF cells and *in vivo* clearance of ^{125}I-fVIII from circulation in mice.

Our studies revealed that the A2 domain of fVIII is responsible for its interaction with LRP, since only A2 domain and HCh, which contains the A2 domain, were able to inhibit the interaction of ^{125}I-fVIII with LRP in a purified system. Thus, it was concluded that A2 is responsible for fVIII binding to LRP. Based on the observation that vWf did not inhibit fVIII binding to LRP, we proposed that LRP may internalize fVIII from its complex with vWf. Indeed, mouse embryonic fibroblasts (MEF) that express LRP, but not fibroblasts genetically deficient in LRP, were able to internalize and degrade ^{125}I-fVIII in the presence of vWf. These processes were competed by RAP and A2 subunit of fVIII, indicating that cellular internalization and degradation were mediated by interaction of the A2 domain of fVIII with LRP. The physiological relevance of the observations utilizing the LRP-expressing cell model system was supported by *in vivo* clearance studies of ^{125}I-fVIII/vWf complex in mice which demonstrated that RAP prolonged the half-life of ^{125}I-fVIII in circulation by 2.5-fold, indicating that a RAP-sensitive receptor, most likely LRP, is responsible for the clearance of fVIII from plasma.

Further localization of the region within the A2 domain responsible for its binding to purified LRP was initiated by the finding that monoclonal antibody with an epitope within A2 domain residues 484-509 completely inhibited fVIII interaction with LRP. Inhibition of fVIII/LRP binding by synthetic peptide with a human fVIII sequence 484-509 indicated that the region of the A2 domain is likely to be directly involved in fVIII binding to purified LRP.

The region 484-509 contains 6 positively charged residues, Lys at positions 493, 496 and 499 and Arg at positions 484, 489 and 490. Basic residues in lipoprotein lipase (Chappell, D.A., *et al.*, *J. Biol. Chem. 268*:14168-14175 (1993)), u-PA-PAI-1 complex (Rodenburg, K.W., *et al.*, *Biochem. J. 329*:55-63 (1998)), and α_2-macroglobulin (Howard, G. C., *et al.*, *J. Biol. Chem 271*:14105-14111 (1996)) were previously shown to be critical for electrostatic interaction with LRP. Alanine substitution of the basic amino acid residues in lipoprotein lipase (Williams, S.E., *et al.*, *J. Biol. Chem. 269*:8653-8658 (1994)), u-PA/PAI-1 complex (Rodenburg, K.W., *et al.*, *Biochem. J. 329*:55-63 (1998)) and in the receptor binding fragment from α_2-macroglobulin (Howard, G.C., *et al.*, *J. Biol. Chem 271*:14105-14111 (1996)) lead to a considerable reduction of affinity for ligand binding to LRP and partial (Rodenburg, K.W., *et al.*, *Biochem. J. 329*:55-63 (1998)) or complete (Howard, G.C., *et al.*, *J. Biol. Chem 271*:14105-14111 (1996)) inhibition of internalization and degradation of the mutants. Therefore, Ala or other amino acid substitutions within the 484-509 region of the recombinant fVIII are useful for reduction of the rate of its LRP-mediated endocytosis and generation of the fVIII mutants with a longer life in the circulation.

FVIII binds to purified LRP with affinity 116 nM, which is much lower than the concentration of fVIII/vWf complex in plasma (1 nM; Wion, K., *et al.*, *Nature 317*:726-730 (1985)). FVIII affinity for LRP is similar to that of the complexes of serine proteases with inhibitors such as ATIII/thrombin (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)), HCII/thrombin and α_1-antitrypsin/trypsin (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)),

which also bind to LRP with affinities 80-120 nM, and weaker than measured for other LRP ligands. It was shown (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)) that internalization and degradation of the above low affinity LRP ligands at their 1 nM concentration by MEF cells occur at a lower rate than that of the u-PA/PAI-1 complex which binds to LRP with high affinity (K_d< 1 nM). Therefore, relatively low affinity of fVIII for LRP is responsible for a slow rate of fVIII internalization and degradation by MEF cells, which is comparable to the rate of ATIII/thrombin, HCII/thrombin and α1-antitrypsin/trypsin degradation at 1 nM concentration of each ligand. The low affinity of fVIII for LRP may also be a necessary requirement for the relatively long fVIII half-life (12-14 h) in plasma of normal individuals (Over, J., *et al.*, *J. Clin. Invest. 62*:223-234 (1978)). Alternatively, the low fVIII affinity for LRP may be compensated by concentration of fVIII molecules on the membrane of LRP-expressing cells, for example, via interaction with cell-surface proteoglycans which have been shown to facilitate the uptake of a number of LRP ligands including lipoprotein lipase (Chappell, D.A., *et al.*, *J. Biol. Chem. 268*:14168-14175 (1993)), hepatic lipase (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)), and thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem. 270*:9543-9549 (1995); Mikhailenko, I., *et al.*, *J. Biol. Chem. 272*:6784-6791 (1997)).

We found that internalization and degradation of isolated fVIII by MEF cells was greater than the corresponding processes for fVIII bound to vWf. In addition, catabolism of the isolated fVIII by MEF cells was only partially inhibited by RAP, indicating that LRP-mediated endocytosis of fVIII is not the sole mechanism of fVIII clearance in the absence of vWf. Our data suggest that in the presence of vWf, which blocks C2 domain-mediated fVIII binding to phospholipid membranes (Saenko, E.L. and Scandella, D., *J. Biol. Chem 270*:13826-13833 (1995)), fVIII binds only to LRP, whereas in the absence of vWf, fVIII binds both to LRP and to an unidentified cell membrane component. The latter binding may lead to fVIII internalization via RAP-independent

pathway, which may be mediated by unidentified receptor as it was previously proposed for hepatic lipase (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)). Since we found that ^{125}I-vWf is not internalized by MEF cells, we propose the model for fVIII endocytosis where fVIII/vWf complex binds to LRP and then vWf dissociates from fVIII during the early stage of fVIII endocytosis, *i.e.* during formation of the coated pits. Since the half-life for the dissociation of fVIII/vWf complex is about 1 hour (Saenko, E.L. and Scandella, D., *J. Biol Chem* 272, 18007-18014 *(1995)*), vWf may delay LRP-mediated endocytosis of fVIII according to the proposed model.

Faster catabolism of fVIII in the absence of vWf is consistent with a demonstrated shorter half-life of fVIII in patients with severe von Willebrand disease (vWD) lacking plasma vWf than that in hemophilia A patients, who have normal levels of vWf (Morfini, M., *et al.*, *Thromb. Haemostas. 70*:270-272 (1993); Lethagen, S., *et al.*, *Ann. Hematol. 65*:253-259 (1992)). Moreover, the half-life of fVIII in vWD patients was prolonged by the presence of vWf in the infused fVIII preparation (Lethagen, S., *et al.*, *Ann. Hematol. 65*:253-259 (1992)). The above observations were previously explained by vWf-mediated stabilization of fVIII by binding to vWf (Wise, R.J., *et al.*, *J. Biol. Chem. 266*:21948-21955 (1991)) and via secondary vWf-mediated release of endogenous fVIII (Wise, R.J., *et al.*, *J. Biol. Chem. 266*:21948-21955 (1991); Kaufman, R.J., *Mol. Cell. Biol. 9*:1233-1242 (1989)). Our data suggest that in addition to the above effects, vWf may reduce the rate of fVIII clearance by preventing LRP-independent pathway and limiting fVIII clearance to LRP-mediated pathway.

The activity of the factor X activation complex (factor Xase), consisting of membrane-bound activated fVIIIa and factor IXa, can be down regulated by inactivation of fVIIIa. The latter occurs via proteolytic degradation of fVIII by activated protein C, factor Xa and factor IXa, and via spontaneous but reversible dissociation of the A2 subunit from fVIIIa heterotrimer (Fay, P. J. and Smudzin, T. M., *J. Biol. Chem 267*:13246-13250 (1992)). Dissociation of the fVIIIa heterotrimer may be accelerated by LRP mediated internalization of the A2

domain, and therefore complement regulation of fVIIIa activity at the sites of coagulation. This hypothesis is supported by availability of LRP at these sites, since LRP is exposed on the surface of monocytes and macrophage (Moestrup, S.K., *et al.*, *Exp. Cell. Res. 190*:195-203 (1990); Moestrup, S.K., *et al.*, *Cell Tissue Res. 269*:375-382 (1992)) and upon vascular injury on fibroblasts and smooth muscle cells (Moestrup, S.K., *et al.*, *Cell Tissue Res. 269*:375-382 (1992)). In addition, it was recently shown that isolated A2 but not isolated A1 and A3-C1-C2 subunits of activated fVIII is able to accelerate factor IXa-catalyzed conversion of factor X by approximately 100-fold (Fay, P.J. and Koshibu, K., *Blood* 92:353a (abstract) (1998)). Even though acceleration of the factor X activation by A2 is only 1 % of that in the presence of heterotrimeric activated fVIII (A1/A2/A3-C1-C2) (Fay, P.J. and Koshibu, K., *Blood* 92:353a (abstract) (1998)), it is possible that LRP-mediated removal of the A2, dissociated from fVIIIa bound to a phospholipid membrane at the site of coagulation, is important to prevent activation of factor X not in the place of the coagulation event.

In summary, the current study demonstrates that LRP can bind fVIII/vWf complex and mediate uptake of fVIII from it. *In vivo* clearance studies underscored the likelihood that LRP indeed functions to remove LRP from plasma.

Example 3

Experiments on the development of recombinant fVIII molecule with extended lifetime in circulation. Since recombinant fVIII products are widely used for fVIII replacement therapy in hemophiliacs who have decreased or nonfunctional fVIII, generation of mutant(s) with a prolonged lifetime is a promising approach to increase the efficacy and reduce the cost of fVIII infusion therapy. A 39 kDa receptor associated protein (RAP) binds reversibly to LRP and inhibits the binding of other ligands and therefore serves as a useful tool for

testing whether LRP is involved in endocytosis of a given ligand. We found that fVIII binding to LRP is inhibited by RAP, confirming the specificity of this interaction. Since von Willebrand factor (vWf), bound to fVIII in the circulation, does not inhibit fVIII binding to purified LRP, we proposed that removal of the fVIII/vWf complex from the circulation may also be LRP-mediated. This role of LRP was supported by our finding that the lifetime of human ^{125}I-fVIII/vWf complex in mice was 2.5-times prolonged in the presence of RAP.

Based on our finding that fVIII amino acids 484-509 were important for fVIII binding to LRP, these amino acids are also important for LRP-mediated endocytosis. To identify the key fVIII amino acids required for endocytosis, single residues 484-509 are mutated to Ala in the B- domain deleted fVIII (B(-) fVIII). Since the basic residues are commonly involved in ligand binding to LRP, six basic residues within 484-509 (3 Lys and 3 Arg) are mutated. U.S. Patent No. 55,859,204 discloses the substitution to Ala of three of these residues (Arg^{484}, Lys^{493} and Arg^{490}); however the other 3 residues – Arg^{490}, Lys^{496} and Lys^{499} – were not substituted. Thus, these residues, individually and in combination, are mutated to Ala. In particular, each of three Arg and each of three Lys are mutated by pairs (this implies preparation of 9 additional fVIII Ala double-mutants).

It is then determined whether endocytosis of the vWf complexes with B(-) fVIII mutant(s) by LRP-expressing cells is reduced compared to that of wild-type B (-) fVIII/vWf. Some mutations result in a decreased rate of internalization and a longer *in vivo* half-life of the complex of the B- fVIII mutant with vWf in plasma of mice compared to that of wild type B- fVIII/vWf complex. The data of the *in vivo* experiments performed in normal and fVIII-deficient mice is mathematically analyzed using biphasic time-course clearance model and equations approximating interspecies scaling which allow to predict fVIII half-life in humans (*Toxicology and Applied Pharmacology* 136:75-78 (1996)).

Clearance of mutant fVIII in vWf-deficient mice which lack fVIII in circulation (a mouse model for severe von Willebrand disease is described in *Proc. Natl. Acad. Sci. USA* 95:9524-9529 (1998)) is also analyzed. These

experiments are aimed at determining mutant fVIII's prolonged half-life in the absence of vWf. Factor VIII interaction with endothelial cells is also analyzed, since this interaction leads to fVIII internalization. In experiments using fluorescent microscopy techniques we observed uptake of fVIII by endothelial cells. Since a fine equilibrium exists in circulation between fVIII bound to vWf and fVIII bound and internalized by endothelial cells, fVIII interaction with phospholipid endothelial cell membrane is an important factor influencing concentration of fVIII (and hence its half-life) in circulation following fVIII injection.

Therefore, individual amino acids within the previously localized fVIII phospholipid binding site (C2 domain region 2303-2332) which play a role in fVIII binding to vWf and to phospholipid are identified. We identify the amino acids playing a key role in fVIII binding to phospholipid, but not to vWf. The amino acids which participate in fVIII binding to vWf and to phospholipids are selected based on the following observations. The homology search between the C2 domain of fVIII and the corresponding region of the discoidin and a family of homologous proteins, containing the so called DS domain, has revealed the fVIII C2 domain sequences involved in the formation of β-structures. In addition, it has been shown that the synthetic fVIII peptide 2310-2320 in which residues 2310 and 2320 are covalently linked to reproduce the corresponding loop structure within the C2 domain, competes for fVIII binding with vWf or phospholipid. Therefore, residues within the 2311-2319 region are mutated to Ala, and other amino acids. Since fV, a fVIII homolog, does not bind to vWf, we mutate only five residues which are unique within the 2311-2319 region of fVIII. The mutants are tested for binding to vWf and phospholipid, which identifies the fVIII residues playing a key role in binding to these ligands.

Clearance of the fVIII mutants with reduced phospholipid binding was compared with that of wt-fVIII in normal and hemophilic mice to determine the contribution of the phospholipid-dependent fVIII clearance component to total fVIII clearance.

The mutations within the C2 domain region 2310-2320 prove to be effective for extension of fVIII lifetime in circulation, so we generate mutant fVIII in which both the C2 domain mutation(s) (positions 2310-2320) and mutation(s) within the A2 (positions 484-509) are combined.

We test the designed extended lifetime fVIII for gene therapy purposes. The extended lifetime fVIII gene is inserted in a virus-based vector, and delivered into hemophilia A mice. The time course of the fVIII *in vivo* expression level is assessed as follows: the number of the gene copies per cell (hepatic), the gene transcription level, fVIII activity and the antigen level are determined. Since it was shown that high titer antibodies increase clearance of fVIII (*Br. J. Hematol. 93*:688-693 (1996)), we examine the immune response against the extended lifetime fVIII. We also compare its half-life in circulation in hemophilia A mice which formed antibodies against wild type fVIII.

What Is Claimed Is:

1. A mutant factor VIII comprising an amino acid substitution at two or more positions in the A2 domain; wherein at least one of said amino acid substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the mutant factor VIII has reduced receptor-dependent clearance; and wherein the mutant factor VIII has procoagulant activity.

2. The mutant factor VIII of claim 1, which lacks the B domain.

3. The mutant factor VIII of claim 2, comprising an amino acid substitution at two or more of positions 484 to 509.

4. The mutant factor VIII of claim 3, comprising an amino acid substitution at two or more of positions 490, 496 or 499.

5. The mutant factor VIII of claim 3, comprising an amino acid substitution at one or more of positions 490, 496 or 499; and at one or more of positions 484, 489 or 493.

6. The mutant factor VIII of claim 5, comprising an amino acid substitution at position 490; and at one or more of positions 484, 489 or 493.

7. The mutant factor VIII of claim 5, comprising an amino acid substitution at position 496; and at one or more of positions 484, 489 or 493.

8. The mutant factor VIII of claim 5, comprising an amino acid substitution at position 499; and at one or more of positions 484, 489 or 493.

9. The mutant factor VIII of claim 2, comprising SEQ ID NO:5.

10. A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 2.

11. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 2.

12. The method of claim 11, which further comprises administering an effective amount of receptor associated protein (RAP).

13. A polynucleotide encoding the mutant factor VIII of claim 2.

14. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 13.

15. The method of claim 14, which further comprises administering an effective amount of a polynucleotide encoding RAP.

16. A mutant factor VIII comprising an amino acid substitution at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the mutant factor VIII has reduced receptor-dependent clearance; and wherein the mutant factor VIII has procoagulant activity.

17. The mutant factor VIII of claim 16, which lacks the B domain.

18. The mutant factor VIII of claim 17, comprising an amino acid substitution at one or more of positions 484 to 509.

19. The mutant factor VIII of claim 18, comprising an amino acid substitution at one or more of positions 490, 496 or 499.

20. The mutant factor VIII of claim 19, comprising an amino acid substitution at position 490.

21. The mutant factor VIII of claim 19, comprising an amino acid substitution at position 496.

22. The mutant factor VIII of claim 19, comprising an amino acid substitution at position 499.

23. The mutant factor VIII of claim 17, comprising SEQ ID NO:5.

24. A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 17.

25. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 17.

26. The method of claim 25, which further comprises administering an effective amount of RAP.

27. A polynucleotide encoding the mutant factor VIII of claim 17.

28. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 27.

29. The method of claim 28, which further comprises administering an effective amount of a polynucleotide encoding RAP.

30. A mutant factor VIII comprising an amino acid substitution at one or more positions in the C2 domain; wherein the mutant factor VIII has reduced receptor-independent clearance; and wherein the mutant factor VIII has procoagulant activity.

31. The mutant factor VIII of claim 30, which lacks the B domain.

32. The mutant factor VIII of claim 31, comprising an amino acid substitution at one or more of positions 2303 to 2332.

33. The mutant factor VIII of claim 32, comprising an amino acid substitution at one or more of positions 2311 to 2319.

34. The mutant factor VIII of claim 31, comprising SEQ ID NO:1.

35. A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 31.

36. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 31.

37. The method of claim 36, which further comprises administering an effective amount of RAP.

38. A polynucleotide encoding the mutant factor VIII of claim 31.

39. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 38.

40. The method of claim 39, which further comprises administering an effective amount of a polynucleotide encoding RAP.

41. A mutant factor VIII comprising:

(i) an amino acid substitution at two or more positions in the A2 domain; wherein at least one of said amino acid substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; and

(ii) an amino acid substitution at one or more positions in the C2 domain as numbered in SEQ ID NO:1;

wherein the mutant factor VIII has reduced clearance; and wherein the mutant factor VIII has procoagulant activity.

42. The mutant factor VIII of claim 41, which lacks the B domain.

43. The mutant factor VIII of claim 42, comprising SEQ ID NO:5.

44. A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 42.

45. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 42.

46. The method of claim 45, which further comprises administering an effective amount of RAP.

47. A polynucleotide encoding the mutant factor VIII of claim 42.

48. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 47.

49. The method of claim 48, which further comprises administering an effective amount of a polynucleotide encoding RAP.

50. A mutant factor VIII comprising:

(i) an amino acid substitution at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; and

(ii) an amino acid substitution at one or more positions in the C2 domain as numbered in SEQ ID NO:1;

wherein the mutant factor VIII has reduced clearance; and wherein the mutant factor VIII has procoagulant activity.

51. The mutant factor VIII of claim 50, which lacks the B domain.

52. The mutant factor VIII of claim 51, comprising SEQ ID NO:5.

53. A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 51.

54. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 51.

55. The method of claim 54, which further comprises administering an effective amount of RAP.

56. A polynucleotide encoding the mutant factor VIII of claim 51.

57. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 56.

58. The method of claim 57, which further comprises administering an effective amount of a polynucleotide encoding RAP.

59. A polypeptide selected from the group consisting of:

(a) a polypeptide comprising a fragment of receptor-associated protein (RAP) which binds LRP;

(b) a polypeptide comprising a mutant of RAP which binds LRP;

(c) a polypeptide comprising an analog of RAP which binds LRP;

(d) a polypeptide comprising 20 contiguous amino acids of the sequence of SEQ ID NO:4, which binds LRP; and

(e) a polypeptide comprising amino acids 203 to 319 of SEQ ID NO:4.

60. A pharmaceutically acceptable composition comprising the polypeptide of claim 59.

61. A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polypeptide of claim 59.

62. The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the A2 domain.

63. The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the C2 domain.

64. The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the A2 domain and an amino acid substitution at one or more positions in the C2 domain.

65. A method of increasing the half-life of factor VIII, selected from the group consisting of:

(a) a method which comprises substituting an amino acid at two or more positions in the A2 domain; wherein at least one of said amino acid substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the resulting factor VIII has reduced receptor-dependent clearance; and wherein the resulting factor VIII has procoagulant activity;

(b) method which comprises substituting an amino acid at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the resulting factor VIII has reduced receptor-dependent clearance; and wherein the resulting factor VIII has procoagulant activity;

(c) a method which comprises substituting an amino acid at one or more positions in the C2 domain; wherein the resulting factor VIII has reduced receptor-independent clearance; and wherein the resulting factor VIII has procoagulant activity;

(d) a method which comprises administering to a patient in need thereof an effective amount of a fragment of RAP, wherein said fragment binds LRP; and

(e) a method comprising two or more of methods (a), (b), (c) or (d).

FIG. 1

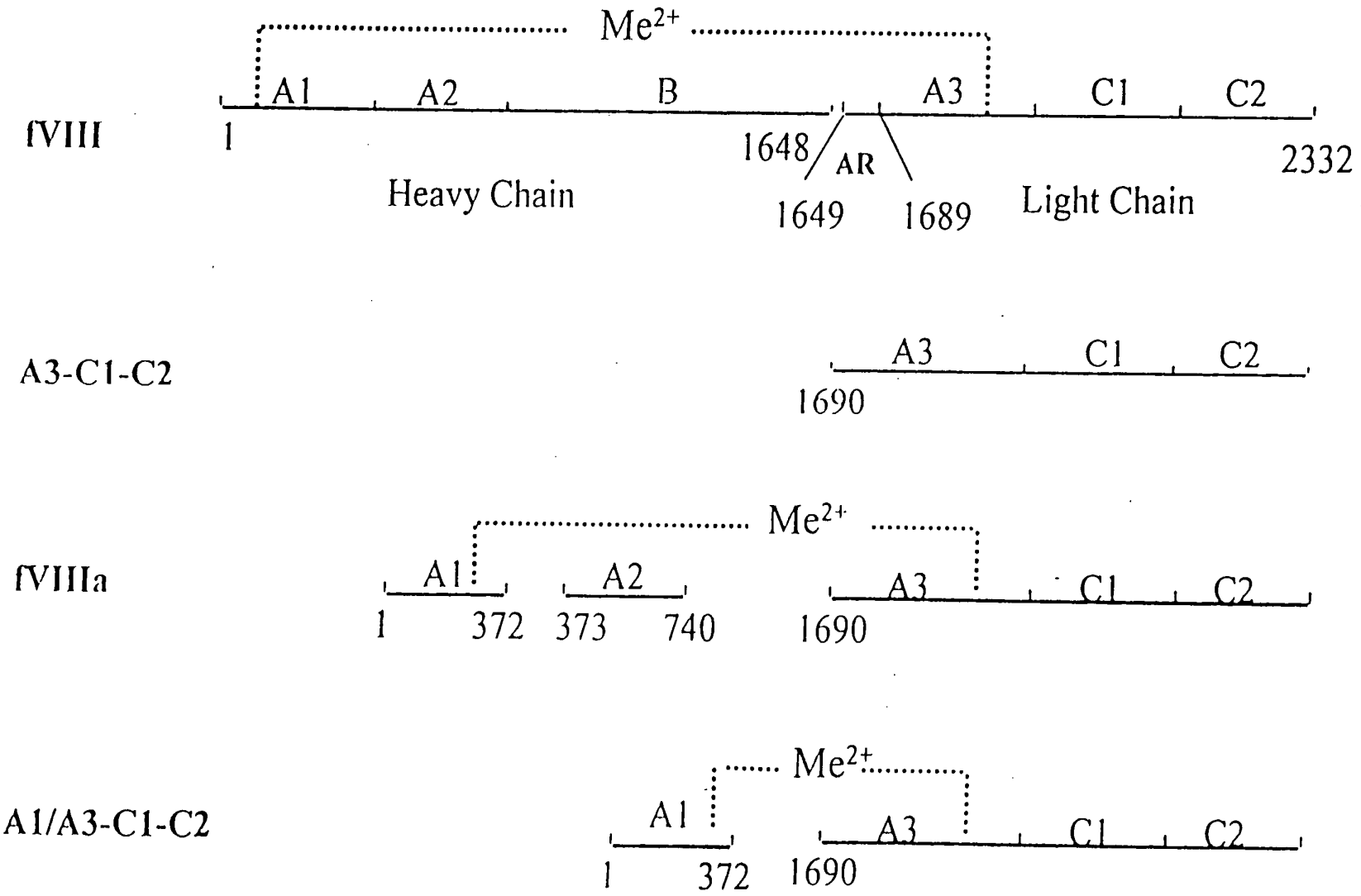

FIG. 2A

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VVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVL

KENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLF

AVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHV

IGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQFLLFCHISSH

QHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIRS

VAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAY

TDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSR
A2 Domain ******
LRP

RLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGL
****
Binding Region

IGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED

PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMV

YEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYED

SYEDISAYLLSKNNAIEPREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQS

PRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQ

FIG. 2B

PLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPR

KNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTN

TLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFH

AINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYN

LYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDF

QITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKF

SSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRL

HPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKAR

LHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQD

GHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLG

CEAQDLY

FIG. 3A

```
   1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL GELPVDARFP PRVPKSFPFN
  61 TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV
 121 GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMASDP LCLTYSYLSH
 181 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD
 241 AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH
 301 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE
 361 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WVHYIAAEEE DWDYAPLVLA
 421 PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL
 481 LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP
 541 TKSDPRCLTR YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR NVILFSVFDE
 601 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL HEVAYWYILS
 661 IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF PFSGETVFMS MENPGLWILG CHNSDFRNRG
 721 MTALLKVSSC DKNTGDYYED SYEDISAYLL SKNNAIEPRS FSQNSRHRST RQKQFNATTI
 781 PENDIEKTDP WFAHRTPMPK IQNVSSSDLL MLLRQSPTPH GLSLSDLQEA KYETFSDDPS
 841 PGAIDSNNSL SEMTHFRPQL HHSGDMVFTP ESGLQLRLNE KLGTTAATEL KKLDFKVSST
 901 SNNLISTIPS DNLAAGTDNT SSLGPPSMPV HYDSQLDTTL FGKKSSPLTE SGGPLSLSEE
 961 NNDSKLLESG LMNSQESSWG KNVSSTESGR LFKGKRAHGP ALLTKDNALF KVSISLLKTN
1021 KTSNNSATNR KTHIDGPSLL IENSPSVWQN ILESDTEFKK VTPLIHDRML MDKNATALRL
1081 NHMSNKTTSS KNMEMVQQKK EGPIPPDAQN PDMSFFKMLF LPESARWIQR THGKNSLNSG
1141 QGPSPKQLVS LGPEKSVEGQ NFLSEKNKVV VGKGEFTKDV GLKEMVFPSS RNLFLTNLDN
1201 LHENNTHNQE KKIQEEIEKK ETLIQENVVL PQIHTVTGTK NFMKNLFLLS TRQNVEGSYD
1261 GAYAPVLQDF RSLNDSTNRT KKHTAHFSKK GEEENLEGLG NQTKQIVEKY ACTTRISPNT
1321 SQQNFVTQRS KRALKQFRLP LEETELEKRI IVDDTSTQWS KNMKHLTPST LTQIDYNEKE
1381 KGAITQSPLS DCLTRSHSIP QANRSPLPIA KVSSFPSIRP IYLTRVLFQD NSSHLPAASY
1441 RKKDSGVQES SHFLQGAKKN NLSLAILTLE MTGDQREVGS LGTSATNSVT YKKVENTVLP
1501 KPDLPKTSGK VELLPKVHIY QKDLFPTETS NGSPGHLDLV EGSLLQGTEG AIKWNEANRP
1561 GKVPFLRVAT ESSAKTPSKL LDPLAWDNHY GTQIPKEEWK SQEKSPEKTA FKKKDTILSL
1621 NACESNHAIA AINEGQNKPE IEVTWAKQGR TERLCSQNPP VLKRHQREIT RTTLQSDQEE
```

FIG. 3B

1681 IDYDDTISVE MKKEDFDIYD EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR

1741 AQSGSVPQFK KVVFQEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI MVTFRNQASR

1801 PYSFYSSLIS YEEDQRQGAE PRKNFVKPNE TKTYFWKVQH HMAPTKDEFD CKAWAYFSDV

1861 DLEKDVHSGL IGPLLVCHTN TLNPAHGRQV TVQEFALFFT IFDETKSWYF TENMERNCRA

1921 PCNIQMEDPT FKENYRFHAI NGYIMDTLPG LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH

1981 VFTVRKKEEY KMALYNLYPG VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC

2041 QTPLGMASGH IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI KVDLLAPMII

2101 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG TLMVFFGNVD SSGIKHNIFN

2161 PPIIARYIRL HPTHYSIRST LRMELMGCDL NSCSMPLGME SKAISDAQIT ASSYFTNMFA

2221 TWSPSKARLH LQGRSNAWRP QVNNPKEWLQ VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL

2281 ISSSQDGHQW TLFFQNGKVK VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM

2341 EVLGCEAQDL Y

FIG. 4

1 MAPRRVRSFL RGLPALLLLL LFLGPWPAAS HGGKYSREKN QPKPSPKRES GEEFRMEKLN
Signal Sequence

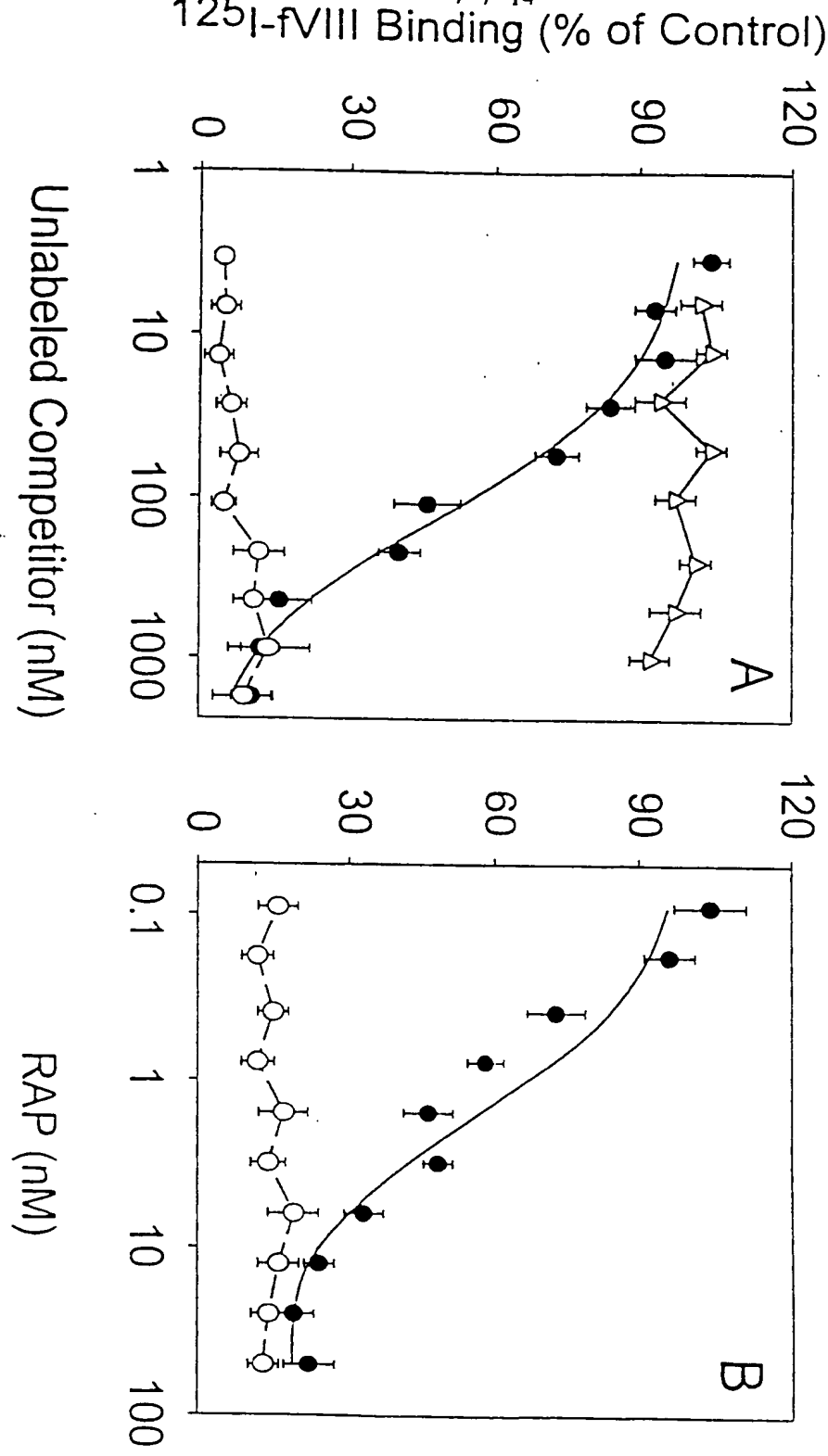
61 QLWEKAQRLH LPPVRLAELH ADLKIQERDE LAWKKLKLDG LDEDGEKEAR LIRNLNVILA

121 KYGLDGKKDA RQVTSNSLSG TQEDGLDDPR LEKLWHKAKT SGKFSGEELD KLWREFLHHK

181 EKVHEYNVLL ETLSRTEEIH ENVISPSDLS DIKGSVLHSR HTELKEKLRS INQGLDRLRR
****

241 VSHQGYSTEA EFEEPRVIDL WDLAQSANLT DKELEAFREE LKHFEAKIEK HNHYQKQLEI
********** ********** ********** ********** ********** **********
LDL Binding Region

301 AHEKLRHAES VGDGERVSRS REKHALLEGR TKELGYTVKK HLQDLSGRIS RARHNEL
********** ********** ********** ********** ********** ***



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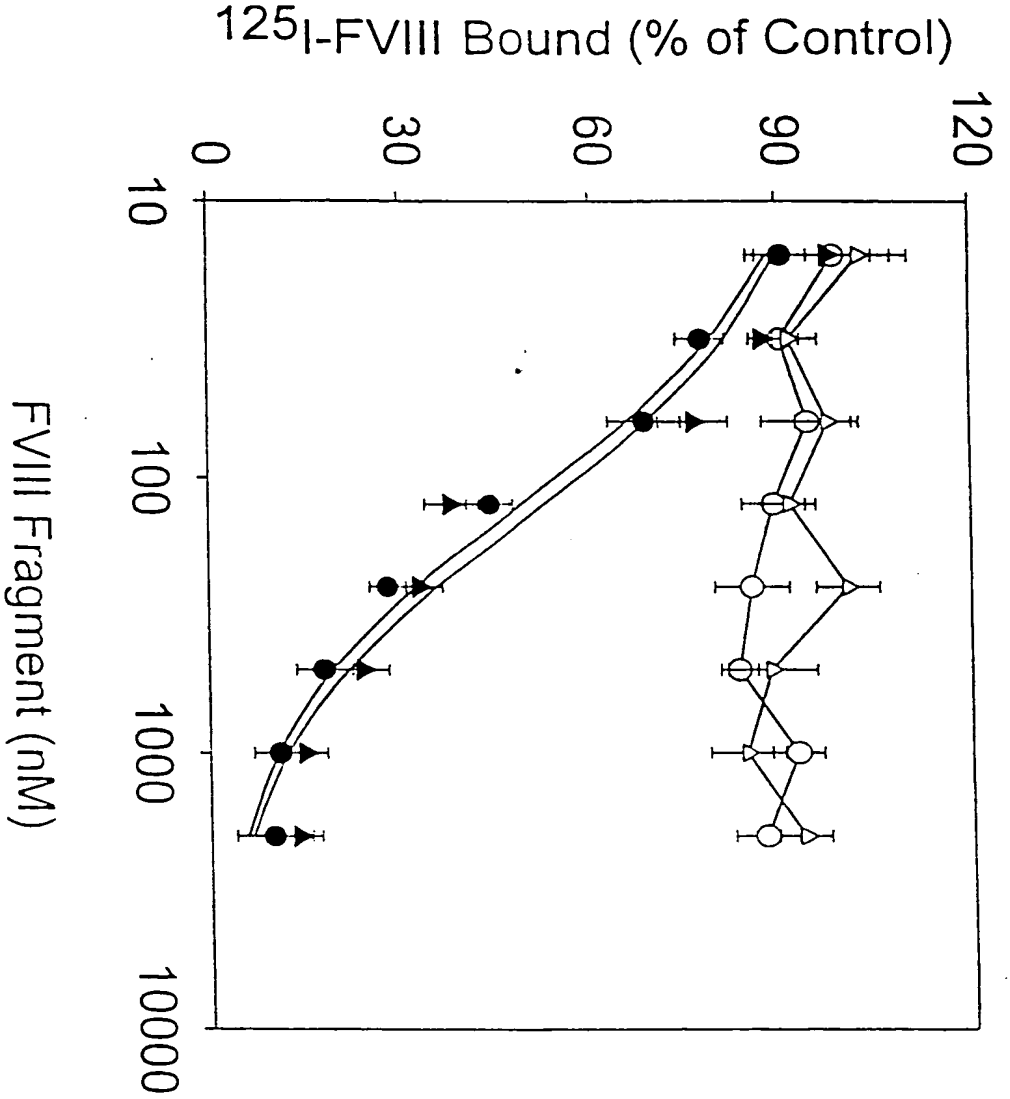
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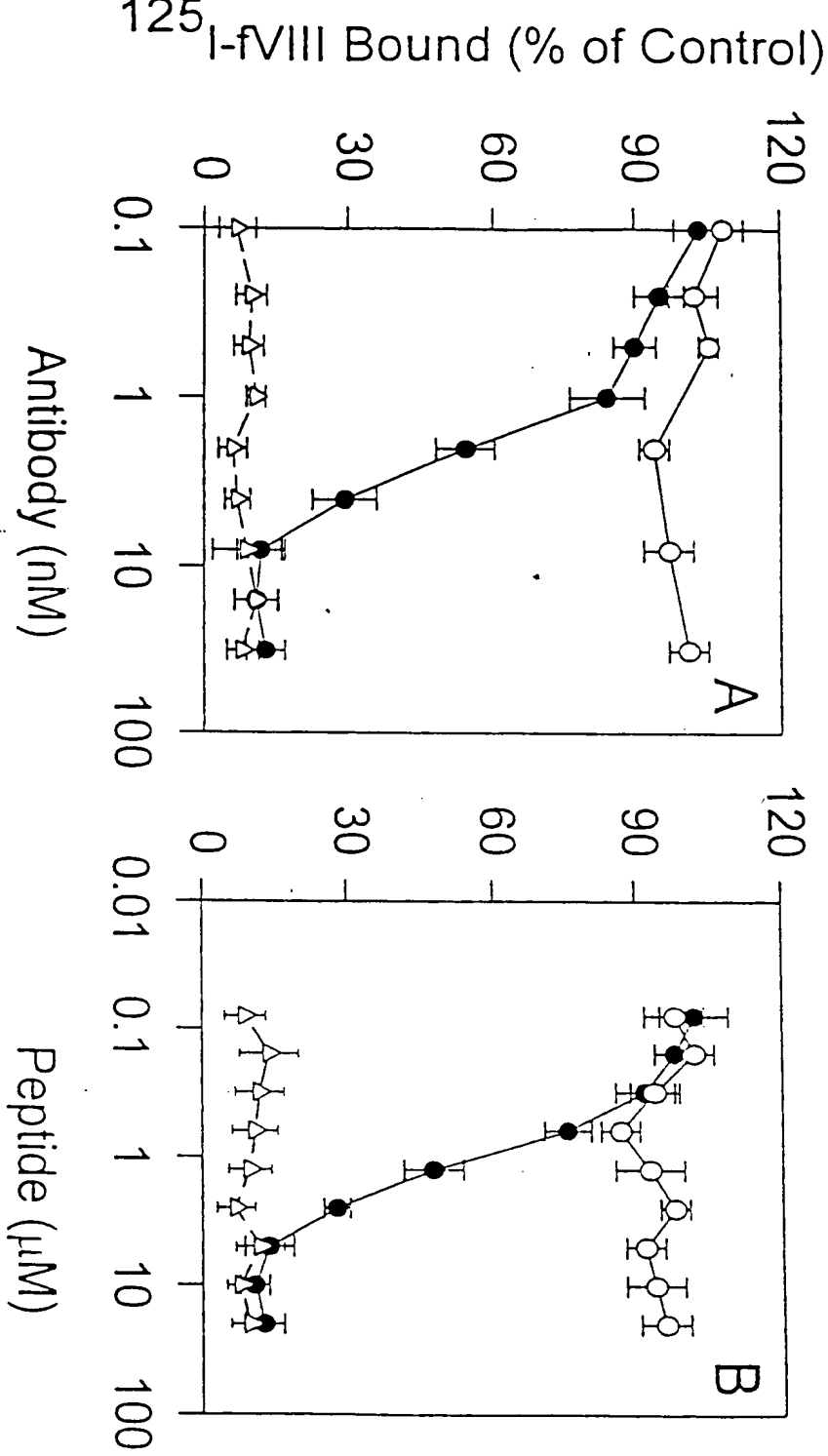
30
60
90
120
FVIII Fragment (nM)
10
100
1000
10000

FIG. 6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0
30
60
90
120
0.1
1
10
100
Antibody (nM)
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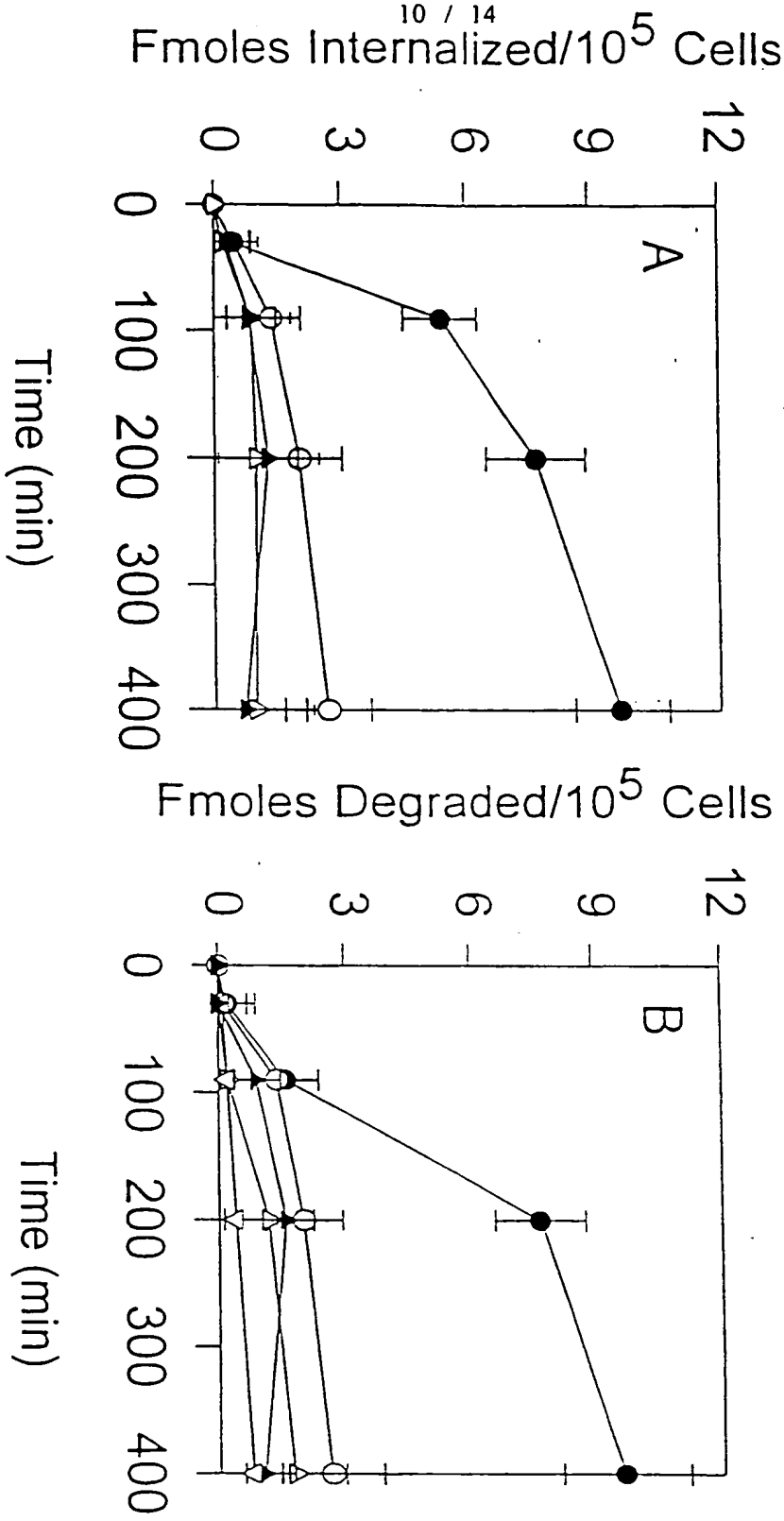
0
30
60
90
120
0.01
0.1
1
10
100
Peptide (μM)
B

FIG. 7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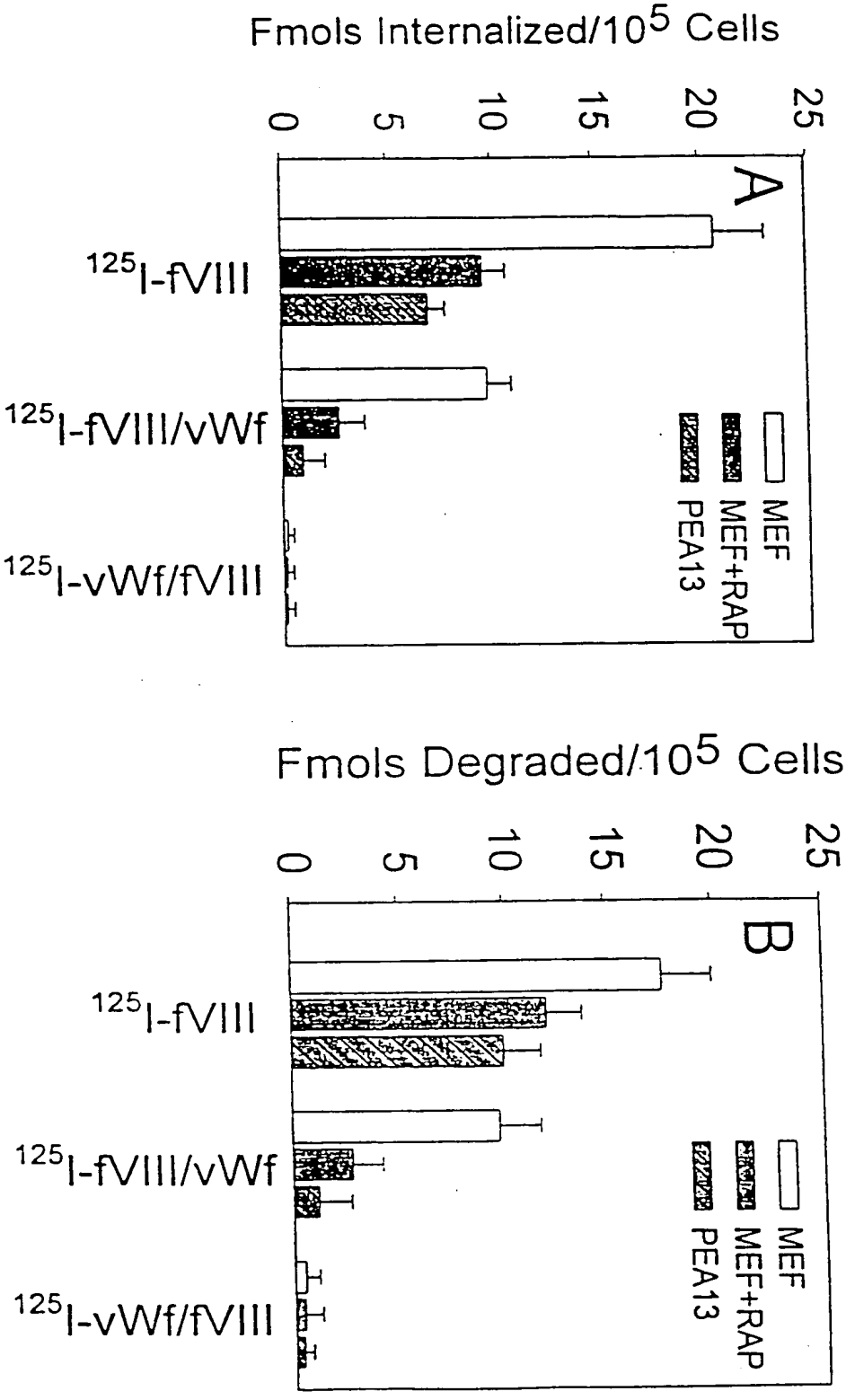
0
3
6
9
12
A
0
100
200
300
400
Time (min)
Fmoles Degraded/10^5 Cells
0
3
6
9
12
B
0
100
200
300
400
Time (min)

FIG. 8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10
15
20
25
A
125I-fVIII
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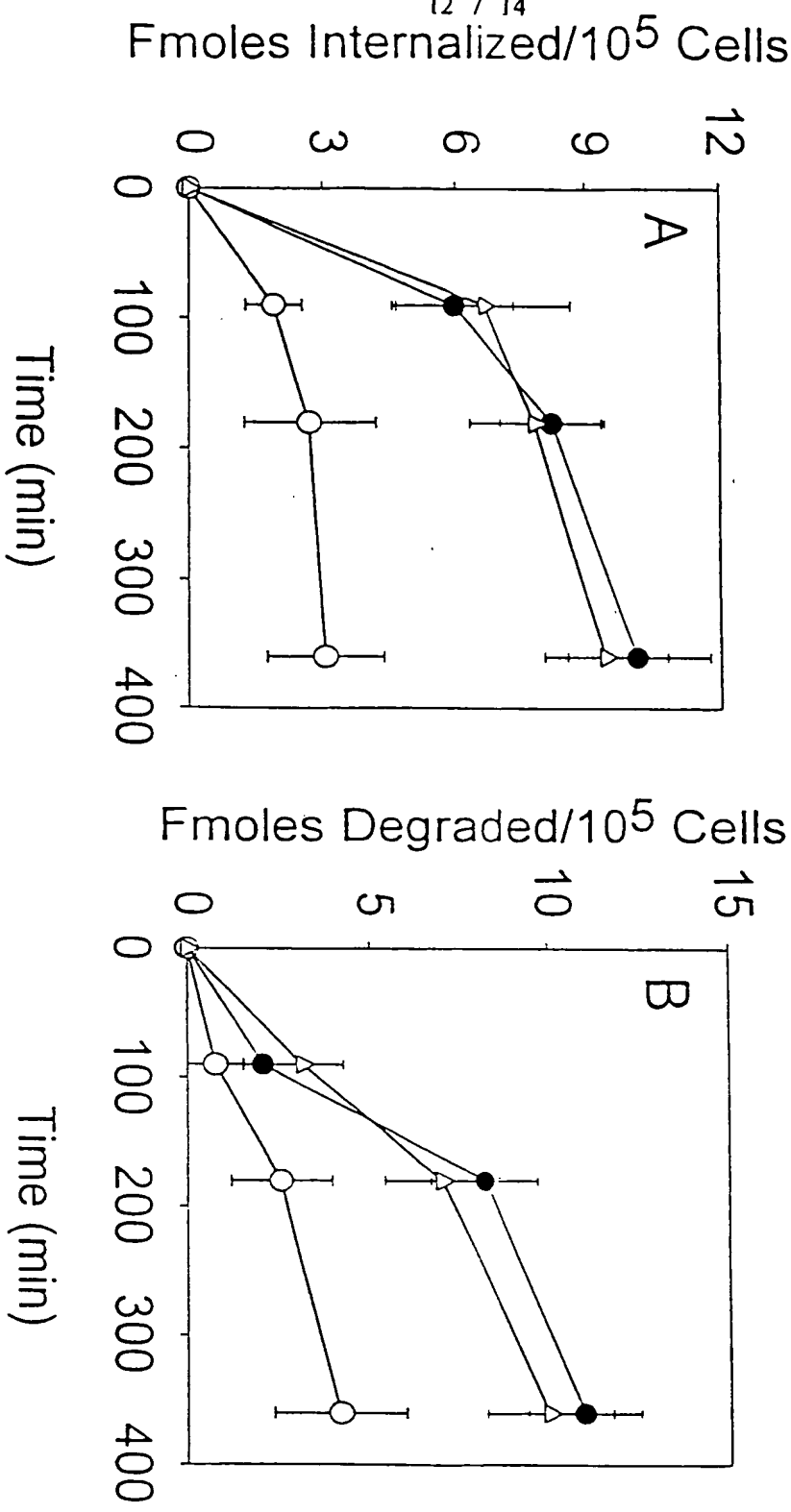
125I-vWf/fVIII
MEF
MEF+RAP
PEA13
Fmols Degraded/10^5 Cells
0
5
10
15
20
25
B
125I-fVIII
125I-fVIII/vWf
125I-vWf/fVIII
MEF
MEF+RAP
PEA13

FIG. 9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3
6
9
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200
300
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Time (min)
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5
10
15
B
0
100
200
300
400
Time (min)

FIG. 10

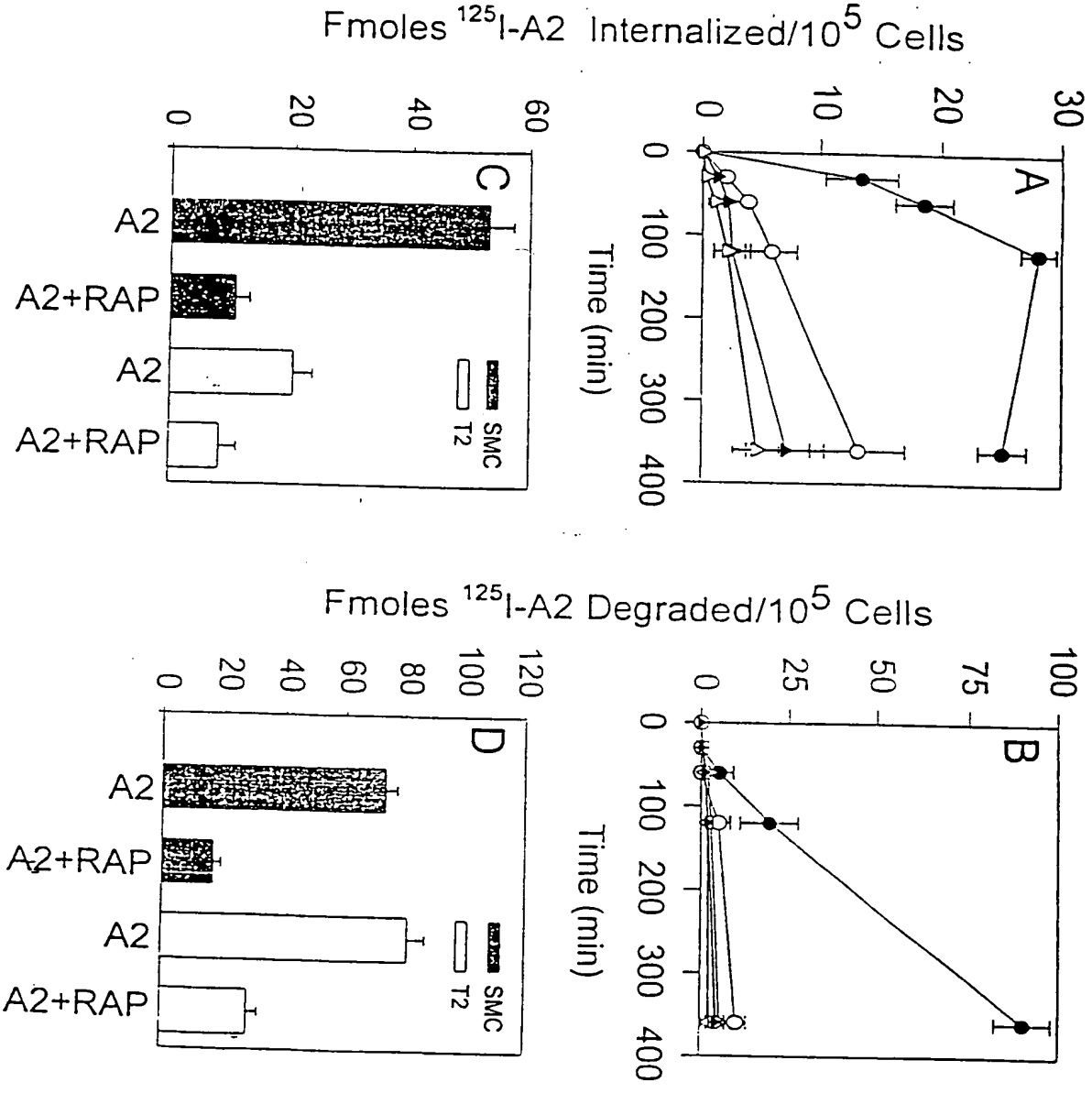

FIG. 11

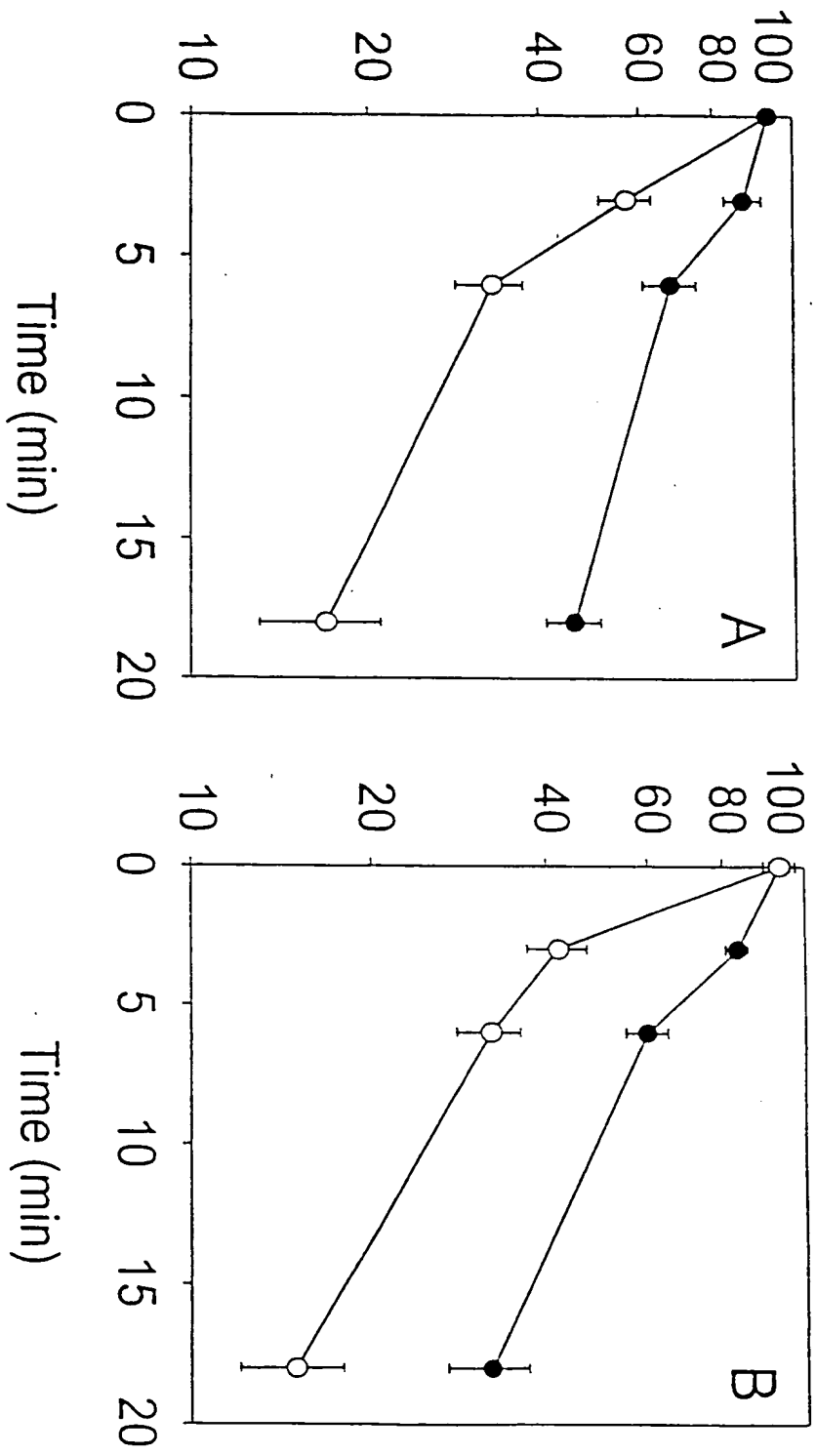

FIG. 12

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Saenko, Evgueni L.
Strickland, Dudley K.

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Met Gln Ile

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        35                  40                  45

act ctg ttt gta gaa ttc acg gat cac ctt ttc aac atc gct aag cca   358
Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
    50                  55                  60

agg cca ccc tgg atg ggt ctg cta ggt cct acc atc cag gct gag gtt   406
Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65                  70                  75                  80

tat gat aca gtg gtc att aca ctt aag aac atg gct tcc cat cct gtc   454
Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85                  90                  95

agt ctt cat gct gtt ggt gta tcc tac tgg aaa gct tct gag gga gct   502
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

gaa tat gat gat cag acc agt caa agg gag aaa gaa gat gat aaa gtc   550
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

ttc cct ggt gga agc cat aca tat gtc tgg cag gtc ctg aaa gag aat   598
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130                 135                 140

ggt cca atg gcc tct gac cca ctg tgc ctt acc tac tca tat ctt tct   646
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160

cat gtg gac ctg gta aaa gac ttg aat tca ggc ctc att gga gcc cta   694
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His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                165                 170                 175

cta gta tgt aga gaa ggg agt ctg gcc aag gaa aag aca cag acc ttg  742
Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180                 185                 190

cac aaa ttt ata cta ctt ttt gct gta ttt gat gaa ggg aaa agt tgg  790
His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195                 200                 205

cac tca gaa aca aag aac tcc ttg atg cag gat agg gat gct gca tct  838
His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210                 215                 220

gct cgg gcc tgg cct aaa atg cac aca gtc aat ggt tat gta aac agg  886
Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240

tct ctg cca ggt ctg att gga tgc cac agg aaa tca gtc tat tgg cat  934
Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                245                 250                 255

gtg att gga atg ggc acc act cct gaa gtg cac tca ata ttc ctc gaa  982
Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

ggt cac aca ttt ctt gtg agg aac cat cgc cag gcg tcc ttg gaa atc 1030
Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275                 280                 285

tcg cca ata act ttc ctt act gct caa aca ctc ttg atg gac ctt gga 1078
Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

cag ttt cta ctg ttt tgt cat atc tct tcc cac caa cat gat ggc atg 1126
Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320

gaa gct tat gtc aaa gta gac agc tgt cca gag gaa ccc caa cta cga 1174
Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                325                 330                 335

atg aaa aat aat gaa gaa gcg gaa gac tat gat gat gat ctt act gat 1222
Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
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            340                 345                 350

tct gaa atg gat gtg gtc agg ttt gat gat gac aac tct cct tcc ttt   1270
Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

atc caa att cgc tca gtt gcc aag aag cat cct aaa act tgg gta cat   1318
Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370                 375                 380

tac att gct gct gaa gag gag gac tgg gac tat gct ccc tta gtc ctc   1366
Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

gcc ccc gat gac aga agt tat aaa agt caa tat ttg aac aat ggc cct   1414
Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

cag cgg att ggt agg aag tac aaa aaa gtc cga ttt atg gca tac aca   1462
Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

gat gaa acc ttt aag act cgt gaa gct att cag cat gaa tca gga atc   1510
Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

ttg gga cct tta ctt tat ggg gaa gtt gga gac aca ctg ttg att ata   1558
Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

ttt aag aat caa gca agc aga cca tat aac atc tac cct cac gga atc   1606
Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

act gat gtc cgt cct ttg tat tca agg aga tta cca aaa ggt gta aaa   1654
Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

cat ttg aag gat ttt cca att ctg cca gga gaa ata ttc aaa tat aaa   1702
His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

tgg aca gtg act gta gaa gat ggg cca act aaa tca gat cct cgg tgc   1750
Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525
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ctg acc cgc tat tac tct agt ttc gtt aat atg gag aga gat cta gct   1798
Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

tca gga ctc att ggc cct ctc ctc atc tgc tac aaa gaa tct gta gat   1846
Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

caa aga gga aac cag ata atg tca gac aag agg aat gtc atc ctg ttt   1894
Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

tct gta ttt gat gag aac cga agc tgg tac ctc aca gag aat ata caa   1942
Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

cgc ttt ctc ccc aat cca gct gga gtg cag ctt gag gat cca gag ttc   1990
Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

caa gcc tcc aac atc atg cac agc atc aat ggc tat gtt ttt gat agt   2038
Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

ttg cag ttg tca gtt tgt ttg cat gag gtg gca tac tgg tac att cta   2086
Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

agc att gga gca cag act gac ttc ctt tct gtc ttc ttc tct gga tat   2134
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

acc ttc aaa cac aaa atg gtc tat gaa gac aca ctc acc cta ttc cca   2182
Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

ttc tca gga gaa act gtc ttc atg tcg atg gaa aac cca ggt cta tgg   2230
Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

att ctg ggg tgc cac aac tca gac ttt cgg aac aga ggc atg acc gcc   2278
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700
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tta ctg aag gtt tct agt tgt gac aag aac act ggt gat tat tac gag   2326
Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

gac agt tat gaa gat att tca gca tac ttg ctg agt aaa aac aat gcc   2374
Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

att gaa cca aga agc ttc tcc cag aat tca aga cac cgt agc act agg   2422
Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740                 745                 750

caa aag caa ttt aat gcc acc aca att cca gaa aat gac ata gag aag   2470
Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

act gac cct tgg ttt gca cac aga aca cct atg cct aaa ata caa aat   2518
Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

gtc tcc tct agt gat ttg ttg atg ctc ttg cga cag agt cct act cca   2566
Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800

cat ggg cta tcc tta tct gat ctc caa gaa gcc aaa tat gag act ttt   2614
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
                805                 810                 815

tct gat gat cca tca cct gga gca ata gac agt aat aac agc ctg tct   2662
Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

gaa atg aca cac ttc agg cca cag ctc cat cac agt ggg gac atg gta   2710
Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
        835                 840                 845

ttt acc cct gag tca ggc ctc caa tta aga tta aat gag aaa ctg ggg   2758
Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850                 855                 860

aca act gca gca aca gag ttg aag aaa ctt gat ttc aaa gtt tct agt   2806
Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865                 870                 875                 880

aca tca aat aat ctg att tca aca att cca tca gac aat ttg gca gca   2854
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Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
                885                 890                 895

ggt act gat aat aca agt tcc tta gga ccc cca agt atg cca gtt cat  2902
Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910

tat gat agt caa tta gat acc act cta ttt ggc aaa aag tca tct ccc  2950
Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
        915                 920                 925

ctt act gag tct ggt gga cct ctg agc ttg agt gaa gaa aat aat gat  2998
Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930                 935                 940

tca aag ttg tta gaa tca ggt tta atg aat agc caa gaa agt tca tgg  3046
Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960

gga aaa aat gta tcg tca aca gag agt ggt agg tta ttt aaa ggg aaa  3094
Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
                965                 970                 975

aga gct cat gga cct gct ttg ttg act aaa gat aat gcc tta ttc aaa  3142
Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990

gtt agc atc tct ttg tta aag aca aac aaa act tcc aat aat tca gca  3190
Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
        995                 1000                1005

act aat aga aag act cac att gat ggc cca tca tta tta att gag aat  3238
Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu Asn
    1010                1015                1020

agt cca tca gtc tgg caa aat ata tta gaa agt gac act gag ttt aaa  3286
Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu Phe Lys
1025                1030                1035                1040

aaa gtg aca cct ttg att cat gac aga atg ctt atg gac aaa aat gct  3334
Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp Lys Asn Ala
                1045                1050                1055

aca gct ttg agg cta aat cat atg tca aat aaa act act tca tca aaa  3382
Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr Ser Ser Lys
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          1060                1065                1070

aac atg gaa atg gtc caa cag aaa aaa gag ggc ccc att cca cca gat   3430
Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile Pro Pro Asp
       1075                1080                1085

gca caa aat cca gat atg tcg ttc ttt aag atg cta ttc ttg cca gaa   3478
Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe Leu Pro Glu
   1090                1095                1100

tca gca agg tgg ata caa agg act cat gga aag aac tct ctg aac tct   3526
Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser
1105                1110                1115                1120

ggc caa ggc ccc agt cca aag caa tta gta tcc tta gga cca gaa aaa   3574
Gly Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys
               1125                1130                1135

tct gtg gaa ggt cag aat ttc ttg tct gag aaa aac aaa gtg gta gta   3622
Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val
           1140                1145                1150

gga aag ggt gaa ttt aca aag gac gta gga ctc aaa gag atg gtt ttt   3670
Gly Lys Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe
       1155                1160                1165

cca agc agc aga aac cta ttt ctt act aac ttg gat aat tta cat gaa   3718
Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu
   1170                1175                1180

aat aat aca cac aat caa gaa aaa aaa att cag gaa gaa ata gaa aag   3766
Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys
1185                1190                1195                1200

aag gaa aca tta atc caa gag aat gta gtt ttg cct cag ata cat aca   3814
Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr
               1205                1210                1215

gtg act ggc act aag aat ttc atg aag aac ctt ttc tta ctg agc act   3862
Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr
           1220                1225                1230

agg caa aat gta gaa ggt tca tat gac ggg gca tat gct cca gta ctt   3910
Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu
       1235                1240                1245
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caa gat ttt agg tca tta aat gat tca aca aat aga aca aag aaa cac 3958
Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys His
1250 1255 1260

aca gct cat ttc tca aaa aaa ggg gag gaa gaa aac ttg gaa ggc ttg 4006
Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu Gly Leu
1265 1270 1275 1280

gga aat caa acc aag caa att gta gag aaa tat gca tgc acc aca agg 4054
Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys Thr Thr Arg
1285 1290 1295

ata tct cct aat aca agc cag cag aat ttt gtc acg caa cgt agt aag 4102
Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr Gln Arg Ser Lys
1300 1305 1310

aga gct ttg aaa caa ttc aga ctc cca cta gaa gaa aca gaa ctt gaa 4150
Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr Glu Leu Glu
1315 1320 1325

aaa agg ata att gtg gat gac acc tca acc cag tgg tcc aaa aac atg 4198
Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn Met
1330 1335 1340

aaa cat ttg acc ccg agc acc ctc aca cag ata gac tac aat gag aag 4246
Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys
1345 1350 1355 1360

gag aaa ggg gcc att act cag tct ccc tta tca gat tgc ctt acg agg 4294
Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg
1365 1370 1375

agt cat agc atc cct caa gca aat aga tct cca tta ccc att gca aag 4342
Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys
1380 1385 1390

gta tca tca ttt cca tct att aga cct ata tat ctg acc agg gtc cta 4390
Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu
1395 1400 1405

ttc caa gac aac tct tct cat ctt cca gca gca tct tat aga aag aaa 4438
Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys
1410 1415 1420

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gat tct ggg gtc caa gaa agc agt cat ttc tta caa gga gcc aaa aaa   4486
Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys
1425                1430                1435                1440

aat aac ctt tct tta gcc att cta acc ttg gag atg act ggt gat caa   4534
Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln
                1445                1450                1455

aga gag gtt ggc tcc ctg ggg aca agt gcc aca aat tca gtc aca tac   4582
Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr
            1460                1465                1470

aag aaa gtt gag aac act gtt ctc ccg aaa cca gac ttg ccc aaa aca   4630
Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr
        1475                1480                1485

tct ggc aaa gtt gaa ttg ctt cca aaa gtt cac att tat cag aag gac   4678
Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp
    1490                1495                1500

cta ttc cct acg gaa act agc aat ggg tct cct ggc cat ctg gat ctc   4726
Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu Asp Leu
1505                1510                1515                1520

gtg gaa ggg agc ctt ctt cag gga aca gag gga gcg att aag tgg aat   4774
Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile Lys Trp Asn
                1525                1530                1535

gaa gca aac aga cct gga aaa gtt ccc ttt ctg aga gta gca aca gaa   4822
Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val Ala Thr Glu
            1540                1545                1550

agc tct gca aag act ccc tcc aag cta ttg gat cct ctt gct tgg gat   4870
Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu Ala Trp Asp
        1555                1560                1565

aac cac tat ggt act cag ata cca aaa gaa gag tgg aaa tcc caa gag   4918
Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu
    1570                1575                1580

aag tca cca gaa aaa aca gct ttt aag aaa aag gat acc att ttg tcc   4966
Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser
1585                1590                1595                1600

ctg aac gct tgt gaa agc aat cat gca ata gca gca ata aat gag gga   5014
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Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly
                1605                1610                1615

caa aat aag ccc gaa ata gaa gtc acc tgg gca aag caa ggt agg act   5062
Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr
            1620                1625                1630

gaa agg ctg tgc tct caa aac cca cca gtc ttg aaa cgc cat caa cgg   5110
Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg
        1635                1640                1645

gaa ata act cgt act act ctt cag tca gat caa gag gaa att gac tat   5158
Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
    1650                1655                1660

gat gat acc ata tca gtt gaa atg aag aag gaa gat ttt gac att tat   5206
Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr
1665                1670                1675                1680

gat gag gat gaa aat cag agc ccc cgc agc ttt caa aag aaa aca cga   5254
Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
                1685                1690                1695

cac tat ttt att gct gca gtg gag agg ctc tgg gat tat ggg atg agt   5302
His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
            1700                1705                1710

agc tcc cca cat gtt cta aga aac agg gct cag agt ggc agt gtc cct   5350
Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
        1715                1720                1725

cag ttc aag aaa gtt gtt ttc cag gaa ttt act gat ggc tcc ttt act   5398
Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
    1730                1735                1740

cag ccc tta tac cgt gga gaa cta aat gaa cat ttg gga ctc ctg ggg   5446
Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
1745                1750                1755                1760

cca tat ata aga gca gaa gtt gaa gat aat atc atg gta act ttc aga   5494
Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
                1765                1770                1775

aat cag gcc tct cgt ccc tat tcc ttc tat tct agc ctt att tct tat   5542
Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr
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          1780                1785                1790

gag gaa gat cag agg caa gga gca gaa cct aga aaa aac ttt gtc aag   5590
Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
       1795                1800                1805

cct aat gaa acc aaa act tac ttt tgg aaa gtg caa cat cat atg gca   5638
Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
   1810                1815                1820

ccc act aaa gat gag ttt gac tgc aaa gcc tgg gct tat ttc tct gat   5686
Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
1825                1830                1835                1840

gtt gac ctg gaa aaa gat gtg cac tca ggc ctg att gga ccc ctt ctg   5734
Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
                1845                1850                1855

gtc tgc cac act aac aca ctg aac cct gct cat ggg aga caa gtg aca   5782
Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr
          1860                1865                1870

gta cag gaa ttt gct ctg ttt ttc acc atc ttt gat gag acc aaa agc   5830
Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
       1875                1880                1885

tgg tac ttc act gaa aat atg gaa aga aac tgc agg gct ccc tgc aat   5878
Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
   1890                1895                1900

atc cag atg gaa gat ccc act ttt aaa gag aat tat cgc ttc cat gca   5926
Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala
1905                1910                1915                1920

atc aat ggc tac ata atg gat aca cta cct ggc tta gta atg gct cag   5974
Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln
                1925                1930                1935

gat caa agg att cga tgg tat ctg ctc agc atg ggc agc aat gaa aac   6022
Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn
          1940                1945                1950

atc cat tct att cat ttc agt gga cat gtg ttc act gta cga aaa aaa   6070
Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
       1955                1960                1965
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gag gag tat aaa atg gca ctg tac aat ctc tat cca ggt gtt ttt gag   6118
Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu
   1970                1975                1980

aca gtg gaa atg tta cca tcc aaa gct gga att tgg cgg gtg gaa tgc   6166
Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys
1985                1990                1995                2000

ctt att ggc gag cat cta cat gct ggg atg agc aca ctt ttt ctg gtg   6214
Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val
                2005                2010                2015

tac agc aat aag tgt cag act ccc ctg gga atg gct tct gga cac att   6262
Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile
           2020                2025                2030

aga gat ttt cag att aca gct tca gga caa tat gga cag tgg gcc cca   6310
Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
       2035                2040                2045

aag ctg gcc aga ctt cat tat tcc gga tca atc aat gcc tgg agc acc   6358
Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr
   2050                2055                2060

aag gag ccc ttt tct tgg atc aag gtg gat ctg ttg gca cca atg att   6406
Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile
2065                2070                2075                2080

att cac ggc atc aag acc cag ggt gcc cgt cag aag ttc tcc agc ctc   6454
Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu
                2085                2090                2095

tac atc tct cag ttt atc atc atg tat agt ctt gat ggg aag aag tgg   6502
Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
           2100                2105                2110

cag act tat cga gga aat tcc act gga acc tta atg gtc ttc ttt ggc   6550
Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly
       2115                2120                2125

aat gtg gat tca tct ggg ata aaa cac aat att ttt aac cct cca att   6598
Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
   2130                2135                2140
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att gct cga tac atc cgt ttg cac cca act cat tat agc att cgc agc   6646
Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
2145                2150                2155                2160

act ctt cgc atg gag ttg atg ggc tgt gat tta aat agt tgc agc atg   6694
Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
                2165                2170                2175

cca ttg gga atg gag agt aaa gca ata tca gat gca cag att act gct   6742
Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala
            2180                2185                2190

tca tcc tac ttt acc aat atg ttt gcc acc tgg tct cct tca aaa gct   6790
Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
        2195                2200                2205

cga ctt cac ctc caa ggg agg agt aat gcc tgg aga cct cag gtg aat   6838
Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn
    2210                2215                2220

aat cca aaa gag tgg ctg caa gtg gac ttc cag aag aca atg aaa gtc   6886
Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val
2225                2230                2235                2240

aca gga gta act act cag gga gta aaa tct ctg ctt acc agc atg tat   6934
Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
                2245                2250                2255

gtg aag gag ttc ctc atc tcc agc agt caa gat ggc cat cag tgg act   6982
Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr
            2260                2265                2270

ctc ttt ttt cag aat ggc aaa gta aag gtt ttt cag gga aat caa gac   7030
Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
        2275                2280                2285

tcc ttc aca cct gtg gtg aac tct cta gac cca ccg tta ctg act cgc   7078
Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg
    2290                2295                2300

tac ctt cga att cac ccc cag agt tgg gtg cac cag att gcc ctg agg   7126
Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
2305                2310                2315                2320

atg gag gtt ctg ggc tgc gag gca cag gac ctc tac tgagggtggc        7172
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Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
2325 2330

cactgcagca cctgccactg ccgtcacctc tccctcctca gctccagggc agtgtccctc 7232

cctggcttgc cttctacctt tgtgctaaat cctagcagac actgccttga agcctcctga 7292

attaactatc atcagtcctg catttctttg gtgggggcc aggagggtgc atccaattta 7352

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gtcacaatcc acaaatgatg caggtgcaaa tggtttatag ccctgtgaag ttcttaaagt 8732

ttagaggcta acttacagaa atgaataagt tgttttgttt tatagcccgg tagaggagtt 8792

aaccccaaag gtgatatggt tttatttcct gttatgttta acttgataat cttatttgg 8852

cattcttttc ccattgacta tatacatctc tatttctcaa atgttcatgg aactagctct 8912

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Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe
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Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser
        -1   1                5                  10

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
    15                  20                  25

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
30                  35                  40                  45

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
                50                  55                  60

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
            65                  70                  75

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
        80                  85                  90
```

His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
95 100 105

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
110 115 120 125

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
130 135 140

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
145 150 155

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
160 165 170

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
175 180 185

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
190 195 200 205

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
210 215 220

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
225 230 235

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
240 245 250

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
255 260 265

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
270 275 280 285

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
290 295 300

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
305 310 315

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
320 325 330

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Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
    335                 340                 345

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
350                 355                 360                 365

Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
                370                 375                 380

Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro
            385                 390                 395

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
        400                 405                 410

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
    415                 420                 425

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
430                 435                 440                 445

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
                450                 455                 460

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
            465                 470                 475

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
        480                 485                 490

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
    495                 500                 505

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
510                 515                 520                 525

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
                530                 535                 540

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
            545                 550                 555

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
        560                 565                 570
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Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
575 580 585

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
590 595 600 605

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
610 615 620

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
625 630 635

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
640 645 650

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
655 660 665

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
670 675 680 685

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
690 695 700

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
705 710 715

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
720 725 730

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg
735 740 745

Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp
750 755 760 765

Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys
770 775 780

Ile Gln Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser
785 790 795

Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr
800 805 810

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Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn
    815                 820                 825

Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly
830                 835                 840                 845

Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu
                850                 855                 860

Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys
            865                 870                 875

Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn
        880                 885                 890

Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met
    895                 900                 905

Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys
910                 915                 920                 925

Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu
                930                 935                 940

Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu
            945                 950                 955

Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
        960                 965                 970

Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala
    975                 980                 985

Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn
990                 995                1000                1005

Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu
                1010                1015                1020

Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr
            1025                1030                1035

Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
        1040                1045                1050
```

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr
1055 1060 1065

Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile
070 1075 1080 1085

Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe
1090 1095 1100

Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser
1105 1110 1115

Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly
1120 1125 1130

Pro Glu Lys Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys
1135 1140 1145

Val Val Val Gly Lys Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu
150 1155 1160 1165

Met Val Phe Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn
1170 1175 1180

Leu His Glu Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu
1185 1190 1195

Ile Glu Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln
1200 1205 1210

Ile His Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu
1215 1220 1225

Leu Ser Thr Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala
230 1235 1240 1245

Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr
1250 1255 1260

Lys Lys His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu
1265 1270 1275

Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys
1280 1285 1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr Gln
1295 1300 1305

Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr
310 1315 1320 1325

Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser
1330 1335 1340

Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr
1345 1350 1355

Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys
1360 1365 1370

Leu Thr Arg Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro
1375 1380 1385

Ile Ala Lys Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr
390 1395 1400 1405

Arg Val Leu Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr
1410 1415 1420

Arg Lys Lys Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly
1425 1430 1435

Ala Lys Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr
1440 1445 1450

Gly Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser
1455 1460 1465

Val Thr Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu
470 1475 1480 1485

Pro Lys Thr Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr
1490 1495 1500

Gln Lys Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His
1505 1510 1515

Leu Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile
1520 1525 1530

Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val
1535 1540 1545

Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu
550 1555 1560 1565

Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys
1570 1575 1580

Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr
1585 1590 1595

Ile Leu Ser Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile
1600 1605 1610

Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln
1615 1620 1625

Gly Arg Thr Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg
630 1635 1640 1645

His Gln Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu
1650 1655 1660

Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe
1665 1670 1675

Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys
1680 1685 1690

Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr
1695 1700 1705

Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly
710 1715 1720 1725

Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly
1730 1735 1740

Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly
1745 1750 1755

Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
1760 1765 1770

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Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu
   1775                1780                1785

Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn
790                1795                1800                1805

Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His
               1810                1815                1820

His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr
            1825                1830                1835

Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly
       1840                1845                1850

Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg
   1855                1860                1865

Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu
870                1875                1880                1885

Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
               1890                1895                1900

Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg
            1905                1910                1915

Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val
       1920                1925                1930

Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser
   1935                1940                1945

Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val
950                1955                1960                1965

Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly
               1970                1975                1980

Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg
            1985                1990                1995

Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu
       2000                2005                2010
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Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser
2015 2020 2025

Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln
030 2035 2040 2045

Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala
2050 2055 2060

Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala
2065 2070 2075

Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe
2080 2085 2090

Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly
2095 2100 2105

Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val
110 2115 2120 2125

Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn
2130 2135 2140

Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser
2145 2150 2155

Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser
2160 2165 2170

Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln
2175 2180 2185

Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro
190 2195 2200 2205

Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro
2210 2215 2220

Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr
2225 2230 2235

Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr
2240 2245 2250

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Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His
   2255                2260                2265

Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly
270                2275                2280                2285

Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu
               2290                2295                2300

Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile
            2305                2310                2315

Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
       2320                2325                2330
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               Met Ala Pro Arg Arg Val Arg Ser Phe Leu Arg Gly
                                  -30                 -25

ctc ccg gcg ctg cta ctg ctg ctg ctc ttc ctc ggg ccc tgg ccc gct   97
Leu Pro Ala Leu Leu Leu Leu Leu Leu Phe Leu Gly Pro Trp Pro Ala
        -20                 -15                 -10

gcg agc cac ggc ggc aag tac tcg cgg gag aag aac cag ccc aag ccg   145
Ala Ser His Gly Gly Lys Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro
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-5              -1  1               5                  10

tcc ccg aaa cgc gag tcc gga gag gag ttc cgc atg gag aag ttg aac   193
Ser Pro Lys Arg Glu Ser Gly Glu Glu Phe Arg Met Glu Lys Leu Asn
                15                  20                  25

cag ctg tgg gag aag gcc cag cga ctg cat ctt cct ccc gtg agg ctg   241
Gln Leu Trp Glu Lys Ala Gln Arg Leu His Leu Pro Pro Val Arg Leu
            30                  35                  40

gcc gag ctc cac gct gat ctg aag ata cag gag agg gac gaa ctc gcc   289
Ala Glu Leu His Ala Asp Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala
        45                  50                  55

tgg aag aaa cta aag ctt gac ggc ttg gac gaa gat ggg gag aag gaa   337
Trp Lys Lys Leu Lys Leu Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu
    60                  65                  70

gcg aga ctc ata cgc aac ctc aat gtc atc ttg gcc aag tat ggt ctg   385
Ala Arg Leu Ile Arg Asn Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu
75                  80                  85                  90

gac gga aag aag gac gct cgg cag gtg acc agc aac tcc ctc agt ggc   433
Asp Gly Lys Lys Asp Ala Arg Gln Val Thr Ser Asn Ser Leu Ser Gly
                95                  100                 105

acc cag gaa gac ggg ctg gat gac ccc agg ctg gaa aag ctg tgg cac   481
Thr Gln Glu Asp Gly Leu Asp Asp Pro Arg Leu Glu Lys Leu Trp His
            110                 115                 120

aag gcg aag acc tct ggg aaa ttc tcc ggc gaa gaa ctg gac aag ctc   529
Lys Ala Lys Thr Ser Gly Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu
        125                 130                 135

tgg cgg gag ttc ctg cat cac aaa gag aaa gtt cac gag tac aac gtc   577
Trp Arg Glu Phe Leu His His Lys Glu Lys Val His Glu Tyr Asn Val
    140                 145                 150

ctg ctg gag acc ctg agc agg acc gaa gaa atc cac gag aac gtc att   625
Leu Leu Glu Thr Leu Ser Arg Thr Glu Glu Ile His Glu Asn Val Ile
155                 160                 165                 170

agc ccc tcg gac ctg agc gac atc aag ggc agc gtc ctg cac agc agg   673
Ser Pro Ser Asp Leu Ser Asp Ile Lys Gly Ser Val Leu His Ser Arg
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gag gag ccc agg gtg att gac ctg tgg gac ctg gcg cag tcc gcc aac   817
Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn
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ctc acg gac aag gag ctg gag gcg ttc cgg gag gag ctc aag cac ttc   865
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gcg cac gag aag ctg agg cac gca gag agc gtg ggc gac ggc gag cgt   961
Ala His Glu Lys Leu Arg His Ala Glu Ser Val Gly Asp Gly Glu Arg
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gtg agc cgc agc cgc gag aag cac gcc ctg ctg gag ggg cgg acc aag   1009
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Ala Asp Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu
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Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

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Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

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Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
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Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile
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1410 1415 1420

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International Bureau

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PCT

(10) International Publication Number
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(21) International Application Number: PCT/US00/14111

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(25) Filing Language: English

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(30) Priority Data:
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(71) Applicant *(for all designated States except US)*: **THE AMERICAN NATIONAL RED CROSS** [US/US]; 8111 Gatehouse Road, Falls Church, VA 22042 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SAENKO, Evgueni, L.** [RU/US]; 9617 Horizon Run Road, Gaithersburg, MD 20879 (US). **STRICKLAND, Dudley, K.** [US/US]; 4124 Barnsley Lane, Olney, MD 20832 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) Designated States *(national)*: AU, CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

(88) Date of publication of the international search report:
18 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/71714 A3

(54) Title: METHODS OF REDUCING FACTOR VIII CLEARANCE AND COMPOSITIONS THEREFOR

(57) Abstract: The present invention provides methods of increasing the half-life of factor VIII. More specifically, the invention provides methods of increasing the half-life of factor VIII by substituting amino acids in the A2 domain or in the C2 domain of factor VIII or in both domains. It further provides factor VIII mutants produced by these methods. The invention also provides a method of using receptor-associated protein (RAP) to increase the half-life of factor VIII. The invention also provides polynucleotides encoding the mutant factor VIII, polynucleotides encoding RAP, and methods of treating hemophilia using the polypeptides and polynucleotides of the invention.

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 00/14111

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/755 C07K14/705 A61K38/37 A61K38/17
A61K31/70 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 808 901 A (IMMUNO AG) 26 November 1997 (1997-11-26)	1-11,13, 14, 16-25, 27,28, 30-36, 38,39, 41-45, 47,48, 50-54, 56,57,65
Y	page 3, line 2 - line 17 page 4, line 14 - line 31 --- -/--	12,15, 26,29, 37,40, 46,49, 55,58, 62-64

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2000	20/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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Form PCT/ISA/210 (second sheet) (July 1992)

NSDOCID: <WO___0071714A3_I_>

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YAKHYAEV A. ET AL.: "Cellular uptake and degradation of the thrombin activated factor VIII fragments" BLOOD, vol. 90, no. 10, suppl. 1, 15 November 1997 (1997-11-15), page 31A XP000907187 ISSN: 0006-4971	59-61,65
Y	abstract	12,15, 26,29, 37,40, 46,49, 55,58, 62-64

X	WO 95 18827 A (NOVONORDISK AS ;NICOLAISEN ELSE MARIE (DK); PERSSON EGON (SE); EZB) 13 July 1995 (1995-07-13)	1,2, 9-11,13, 14,16, 17, 23-25, 27,28,65
	the whole document	

X	US 5 650 391 A (SCHWARTZ ALAN L ET AL) 22 July 1997 (1997-07-22) column 2, paragraph 1; example 1	59,60

P,X	WO 00 28021 A (BAXTER AKTIENGESELLSCHAFT ;TURECEK PETER (AT); SCHEIFLINGER FRIEDR) 18 May 2000 (2000-05-18)	30,31, 34-40, 59-61, 63,65
	the whole document	

P,X	WO 00 27425 A (BAXTER AKTIENGESELLSCHAFT ;TURECEK PETER (AT); BINDER BERND (AT);) 18 May 2000 (2000-05-18)	59-61,65
P,A	page 1, paragraph 4	12,15, 26,29, 37,40, 46,49, 55,58, 62-64
	page 2, last paragraph -page 3, line 19 page 4, last paragraph -page 5, paragraph 3	

	-/--	

1

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/14111

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SAENKO E. L. ET AL.: "Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 53, 31 December 1999 (1999-12-31), pages 37685-37692, XP002152276 ISSN: 0021-9258 the whole document	1-29, 59-65

1

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11,13,14,16-25,27,41-45,47,48,50-54,56, 57 (completely),12,15,26,29,46,49,55,58,62,64, 65 (partially)

   Mutant factor VIII comprising an amino acid substitution in the A2 domain; polynucleotide encoding said mutant factor VIII; pharmaceutical compositions comprising said mutant factor VIII or said polynucleotide; methods for treating hemophilia; methods of increasing the half-life of factor VIII.

2. Claims: 30-36,38,39 (completely),37,40,63,65 (partially)

   Mutant factor VIII comprising an amino acid substitution in the C2 domain; polynucleotide encoding said mutant factor VIII; pharmaceutical compositions comprising said mutant factor VIII or said polynucleotide; methods for treating hemophilia; methods of increasing the half-life of factor VIII.

3. Claims: 59,60,61(completely),12,15,26,29,37,40,46,49,55,58, 62-65 (partially)

   Polypeptide selected from the group consisting of fragments, mutants or analogs of RAP; pharmaceutical composition comprising said polypeptide, RAP or a polynucleotide encoding RAP; methods for treating hemophilia; methods of increasing the half-life of factor VIII.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims
11,12,14,15,25,26,28,29,36,37,39,40,45,46,48,49,54,55,57,58 and 61-64 as well as claim 65, as far as an in vivo application is concerned, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 00/14111

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0808901 A	26-11-1997	AT 403438 B	25-02-1998
		AT 92196 A	15-07-1997
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		JP 10057060 A	03-03-1998
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